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FINAL REPORT

(6) MICROBIAL DEGRADATION OF PESTICIDES.

by

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MAJOR ACCOMPLISHMENTS

Over 110 marine bacteria were isolated and tested for their ability to convert DDT to water-soluble products. Forty-seven bacteria were found to convert 5% to 10% of the ^{14}C-DDT to water-soluble products, 38 bacteria solubilized less than 5% of the insecticide, and 29 bacteria were apparently inactive by the test method employed. Mucor alternans, a fungus that converted DDT to water-soluble metabolites at a rate four times greater than the most active marine bacterium, was used as a model for determining the identities of the water-soluble products. These compounds were not DDT, DDA, PCPA, or 2-chlorosuccinic acid and do not contain chlorine. Therefore, previously uncharacterized products representing the most extensive microbial degradation of DDT yet reported, are probably formed by M. alternans. The hypothesis that nutrient availability was limiting DDT degradation was tested with model marine communities of sea water containing bottom sediments, to which were added eighty different carbon sources in a total of 100 separate treatments. Water soluble products of DDT metabolism were not detected.

Microorganisms were found to be capable of converting diphenylmethane, an analog of DDT, to 1,1,1',1'-tetraphenyldimethyl ether, and studies were conducted to determine whether microorganisms could carry out the same reaction with the DDT metabolite bis(p-chlorophenyl-benzhydrol). Microorganisms able to grow at the expense of various carbon sources having a structural relationship to DDT were isolated, and the ability of these isolates to dechlorinate DDT, its metabolites and its analogs was tested. During the isolations, it was noted that para-substituted aromatic compounds were toxic to some bacteria, even at low levels, but a few stimulated growth of individual bacteria.

The relationship of chemical structure to biodegradability of DDT analogs was investigated. Para substitution of one of the two aromatic rings with chloro, nitro, hydroxyl, or amino groups significantly reduced the rate of biodegradability. The most resistant compounds were those with both rings containing these substituents.

Pseudomonas putida, an organism capable of utilizing diphenylmethane as sole source of carbon and energy, converted bis(p-chlorophenyl)acetic acid to bis(p-chlorophenyl)methane, 4,4'-dichlorobenzhydrol, and 4,4'-dichlorobenzophenone by cometabolism. The organism also dehalogenated 4,4'-dichlorobenzohydrol and 4,4'-dichlorobenzophenone. This is the first report of such dehalogenations of ring chlorines derived from DDT. Pseudomonas putida was also shown to convert diphenylmethane to benzhydrol and benzophenone. The organism was also found to be capable of ring cleavage of diphenylmethane and benzhydrol, producing phenylacetic and phenylglycolic acids, respectively. Hydroxybenzhydrols and a hydroxylated benzophenone were also obtained as products of benzhydrol cometabolism. A bacterial isolate using phenylacetic acid as sole carbon and energy source cometabolized p-chlorophenylacetic acid, and a product likely to be a monohydroxylated chlorophenylacetic acid was recovered from the culture filtrate.

Studies were conducted to assess the effect of salinity, temperature, oxygen tension and presence of sediment organic nutrients and algal cells on the decomposition of DDT in model marine ecosystems. In the model ecosystems receiving the alga Cylindrospermum sp. or diphenylmethane, DDD, DDE, and DBP were formed from DDT. In addition to DDD and DDE, a compound with the chromatographic characteristics of DBP was detected in waters receiving the insecticide.

DDT and its breakdown products had no significant effect on respiration of microbial communities or algal productivity. Bis(p-chlorophenyl)methane, 4,4'-dichlorobenzhydrol, 4,4'-dichlorobenzophenone, bis(p-chlorophenyl)acetic acid, p-chlorophenylacetic acid and DDT enhanced algal development.

The products formed in the metabolism of DDT by bacteria and fungi were identified as 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, DDM, DBH, and DBP. Several other, as yet uncharacterized, products were also synthesized by these microorganisms. The compounds identified during the degradation of DDT metabolites were: DDM, DBH and DBP from DDA; DBH and DBP from DDM; and DBH from DBP. An important metabolite reported for the first time with fungi is p-chlorophenylacetic acid (PCPA), a ring cleavage product generated in the degradation of DDM. p-Chlorophenylglyoxaldehyde is also reported for the first time as a product of ring cleavage.

Arthrobacter sp., an isolate capable of utilizing phenylacetic acid as sole source of carbon, was tested for its ability to degrade PCPA. Metabolites of retention times 554 and 653 s were detected by gas-liquid chromatography. The product of retention time 653 s was identified as 4-chloro-3-hydroxyphenylacetic acid by using coupled gas-liquid chromatography-mass spectrometry and also be comparing the unknown with the mass spectrum of a sample of authentic 4-chloro-3-hydroxyphenylacetic acid that was synthesized.

Bacteria able to metabolize at least one of 12 organophosphate insecticides as sole phosphorus sources and at least one of 5 carbamate insecticides as sole nitrogen source were isolated from sewage and soil by enrichment-culture techniques. Two microbial isolates, selected for their versatility in metabolizing organophosphate insecticides as sole phosphorus source, were identified as Pseudomonas putida and Pseudomonas sp. (designated Pseudomonas 7, while one isolate selected for its versatility in metabolizing carbamate

insecticides as sole nitrogen source and designated as Pseudomonas 44. The extent of growth (as measured by turbidity) of P. putida and Pseudomonas 7 was linearly related to the level of phosphorus (as KH_2PO_4, diazinon, or malathion) in the medium for a concentration range of 0.03 to 0.15 mM, while for Pseudomonas 44 the extent of growth was linearly related to the level of nitrogen (as $(NH_4)_2SO_4$, baygon or carbaryl) in the medium for a concentration range of 0.15 to 0.75 mM. Maximum optical densities for P. putida and Pseudomonas 7 were 1.45, 0.85, and 1.00 and specific growth rates were 0.31, 0.18, and 0.21 h^{-1} with KH_2PO_4, diazinon, and malathion, respectively. For Pseudomonas 44 maximum optical densities were 1.15, 0.87, 0.84 and specific growth rates were 0.26, 0.19, and 0.18 h^{-1} with $(NH_4)_2SO_4$, baygon, and carbaryl, respectively. When growth (measured as cell protein) of these isolates was correlated with metabolism of the particular insecticide used as phosphorus source (diazinon, malathion) or nitrogen source (baygon, carbaryl) the extent of metabolism approached the theoretical values for the ratios of cell protein:phosphorus and cell protein:nitrogen. Thus, the isolates were not oligophosphorophiles or oligocarbophiles and were not utilizing possible contaminant inorganic phosphate or ammonium as phosphorus or nitrogen source, respectively, in place of the insecticide.

Studies of resting cell suspensions of P. putida and Pseudomonas 7 indicated that both constitutive and induced enzyme systems were responsible for the metabolism of diazinon and malathion, while only an induced enzyme system was responsible for the metabolism of baygon and carbaryl. Enzymes in cell-free extracts, partially purified by $(NH_4)_2SO_4$ fractionation, of the three isolates demonstrated broad substrate specificity toward various organophosphate and carbamate insecticides. Specific activities ranged from 0.22 to 2.01 μM organophosphate disappeared/min/mg protein and 0.29 to 1.98 μM car-

bamate disappeared/min/mg protein. The cell-free extract studies further supported the conclusion that induced and constitutive enzyme systems were responsible for organophosphate and carbamate metabolism by P. putida and Pseudomonas strains 7 and 44.

Breakdown products of various organophosphates from the cell-free extract studies were identified as dimethyl and diethyl phosphate and thiophosphate compounds. Neither P putida not Pseudomonas 7 metabolized ionic dialkyl phosphate and thiophosphate compounds as sole phosphorus source. The occurrence of ionic dialkyl(thio)phosphate suggests that a phosphatase enzyme(s) was responsible for the hydrolysis of the parent organophosphate molecule. Thus, for P. putida and Pseudomonas 7, a major pathway of degradation of organophosphate insecticides may be a hydrolytic attack to liberate the ionic dialkyl(thio)phosphate. The inability of the isolates to metabolize ionic dialkyl phosphate and thiophosphate compounds suggests that these molecules may be more persistent in nature than once thought, and concern about their potential environmental toxicity and fate is justified.

I. Section 1

A. MATERIALS AND METHODS

Media. Marine microorganisms were isolated and maintained on two media. DYE contained 0.05 g K_2HPO_4, 15 g agar, and 1000 ml aged sea water. WHO medium, a general isolation medium for marine microorganisms (24), contained 1.0 g peptone, 2.0 g glucose, 0.05 g K_2HPO_4, 15 g agar, and 1000 ml of aged sea water. Mucor alternans was grown on a glucose-salts medium which contained 5 g glucose, 1 g NH_4NO_3, 1 g K_2HPO_4, 1 g KH_2PO_4, 0.2 g $MgSO_4$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.01 g Fe_2SO_4 and 1000 ml distilled water (4).

Gas-liquid chromatography. A Varian 200 gas-chromatograph equipped with a flame ionization detector and two different columns and operating conditions was employed. When a 10% DC 200 column on chromosorb W-AW was used, the operating temperatures were 185 C, column; 215 C, injector; and 195 C, detector. The second column was a 15% SE 30 on chromosorb W, and the operating temperatures when it was used were 90 C, column; 185 C, injector; and 215 C, detector. The flow rate of N_2 through the columns was 45 ml/min.

Esterification using diazomethane. The method of Schlenk and Gellerman (21) was used to esterify acids prior to gas chromatography. The diazomethane generator resembled one described by these authors. As soon as excess CH_2N_2 was seen in the sample (the sample yellowed), the sample tube was disconnected, and its contents were evaporated to dryness immediately with a flow of N_2. The esterified acids were then dissolved in chloroform or ether and examined directly by thin-layer chromatography (TLC) or gas-liquid chromatography (GLC).

Thin-layer chromatography. The chemicals were spotted on Eastman Chromatogram Sheet 6060 Silica Gel bearing a fluorescent indicator. After development, the spots were visualized with a UVS 12 Mineralight (Ultraviolet Products, Inc., San Gabriel, Calif.). Chromatograms to be counted for their radioactivity were cut into 4-cm strips and counted with an Actigraph III strip counter (Nuclear-Chicago Corp.).

Scintillation counting. Samples of 0.1 to 0.5 ml were placed into scintillation vials, and 10 to 15 ml of Bray's scintillation fluid (5) was added. The vials were counted with a liquid scintillation counter (Nuclear-Chicago Corp. model Mark I) which automatically subtracted the background counts.

Chemicals. 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), 2,2-bis(p-chlorophenyl)acetic acid (DDA), 4,4'-dichlorobenzophenone (DBP), 4,4'-dichlorobenzhydrol (DBH), and p-chlorophenylacetic acid (PCPA) were obtained from Aldrich Chemical Co., Milwaukee, Wis. 2-Chlorosuccinic acid was provided by K and K Laboratories, Plainview, N. Y. Hexanes used for extractions were pesticide grade from Fisher Scientific Co., Fairlawn, N. J. Column packing materials were purchased from Applied Science Laboratories, State College, Pa.

B. RESULTS

Isolation of microorganisms capable of degrading DDT. Samples of sea water and sediment materials were collected from seven sites along the Connecticut coast. The seven sites included four distinct habitats: brackish river water, salt marsh, and areas with bottoms consisting of fine black sand or course gravel. Samples were collected in sterile containers and they then were shaken vigorously and streaked onto DYE and WHO agar within 2 hr of collection. The plates were allowed to incubate at about 23 C. Different colony types were picked from each plate, and pure cultures were obtained by standard techniques.

Each of the isolates was examined for its ability to transform DDT to water-soluble products. To measure this conversion, each bacterium was grown on 0.2% yeast extract-sea water medium in the presence of ^{14}C-labeled DDT. The final concentration of DDT was 0.5 μg/ml and its specific activity was 100 μc/44.4 mg. After 7 to 18 days of incubation at 23 C, the cells were removed by centrifugation, and DDT and most of its non-polar products were extracted from the supernatant fluid with hexane. The water phase was then examined for residual radioactivity.

About 48 of the bacteria were found to have converted 5 to 10% of the ^{14}C-DDT to water-soluble products, 38 solubilized less than 5% of the insecticide, and 29 were apparently inactive by the test methods employed. The results summarized in Table I show that four areas contained a particularly high percentage of active organisms. They were Oyster Creek surface water, Kelsey Point surface water, Sagamore Terrace surface water, and Sagamore Terrace gravel, where 70, 66, 60, and 64% of the isolates were active, respectively. The most striking observation from these somewhat

Table 1. The ability of marine isolates to solubilize ^{14}C-DDT.

Sample Area	Surface			Sediment		
	No. tested	No. active[a]	%[b]	No. tested	No. active[a]	%[b]
Connecticut River at Saybrook, Conn. (brackish)	16	5	31	11	4	36
Saybrook salt marsh	8	1	12	7	2	28
Coast 1 mi from Conn. River	14	6	43	-	-	-
Coast 2 mi from Conn. River	9	4	44	-	-	-
Oyster Creek (brackish)	10	7	70	5	2	40
Kelsey Point (fine black sand)	3	2	66	11	4	36
Sagamore Terrace (course gravel)	5	3	60	11	7	64

[a] The number of DDT solubilizers was considered to be those isolates which converted more than 5% of the added labeled DDT to water-soluble products.

[b] Percent of the total number of bacteria tested from that site which solubilized DDT.

randomly chosen isolates was the fact that a large percentage of the isolates had the ability to solubilize DDT and presumably to initiate its degradation.

Water-soluble products formed by Mucor alternans.

A strain of Mucor alternans which was exceptionally active in converting DDT to water-soluble products was isolated by Anderson and Lichtenstein (2). This fungus was used as a model to characterize more completely the pathway of DDT degradation. The fungus was grown in 500 ml of a glucose-salts medium containing 2 μg DDT/ml including 2.9 μc ^{14}C-DDT as described by Anderson and Lichtenstein (3). The mycelium was removed from the medium by filtration after 7 days of incubation and the filtrate was extracted three times with 500 ml portions of hexane each time. The first method of isolation is outlined in Fig. 1. The radioactivity recovered at each step in the procedure is also given.

Analysis of the compounds thus generated microbiologically from DDT, using both thin-layer and gas-liquid chromatography, is still in progress. In Table 2 is a summary of the data. From these data, it is evident that the water-soluble degradation products were not DDT, DDA, DBH, DBP, PCPA or 2-chlorosuccinic acid. Previous work in this laboratory has shown that PCPA is formed microbiologically from DDT (9,17), and thus PCPA or one or more of the other compounds also could have been the water-soluble metabolites synthesized from DDT by M. alternans. Therefore, the soluble products appear to be new metabolites generated microbiologically from DDT.

Methylation of these compounds with diazomethane altered their mobility somehwat. When the products were chromatographed on TLC plates

Figure 1. Extraction procedure used. From Anderson and Lichtenstein (3).

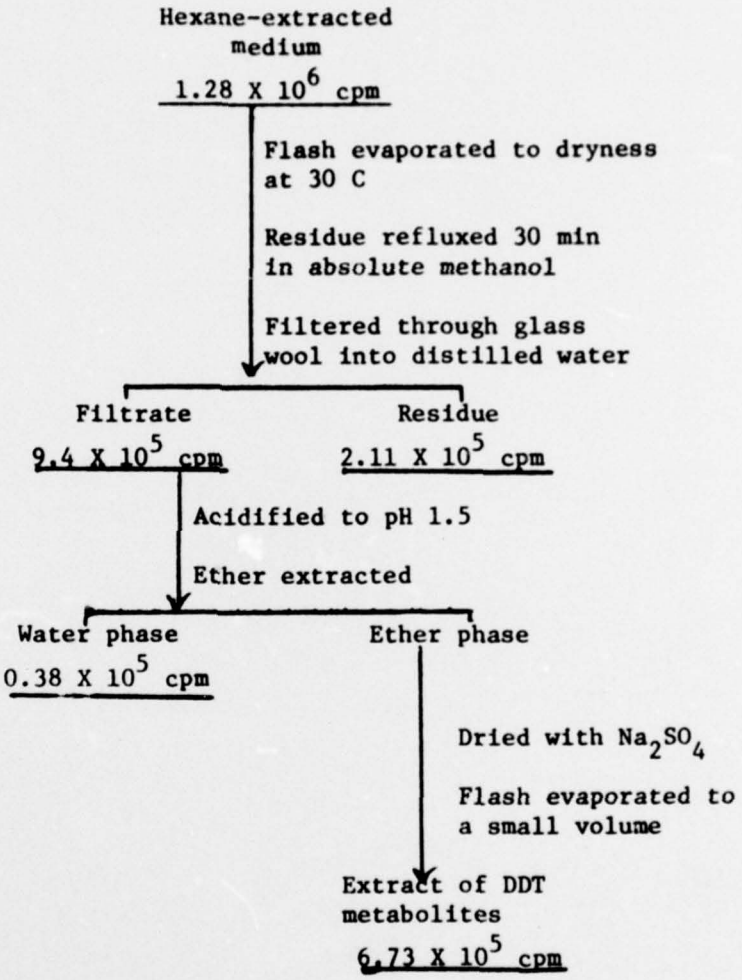


Table 2. Analysis of water-soluble products of Mucor alternans.

Compound	Retention time (GLC), min		R_f value (TLC)	
	10% DC 200	15% SE 30	Solvent I[d]	Solvent II[e]
DDT	71:00	-	-	0.94
DDA-O-Me[a]	34:00	-	0.82	-
PCPA-O-Me	2:12	-	0.90	-
Mucor alternans-O-Me[b]	2:10	-[c]	0.00	0.00
2-Chlorosuccinic acid	-	15:30	-	-
DDA	-	-	-	0.09
DBH	-	-	-	0.14
DBP	-	-	-	0.74
PCPA	-	-	-	0.19

[a] -O-Me indicates that the acid was esterified with diazomethane.

[b] The M. alternans extract was treated with diazomethane.

[c] None of the peaks corresponded to the standards.

[d] Solvent was acetone:hexane (10:90).

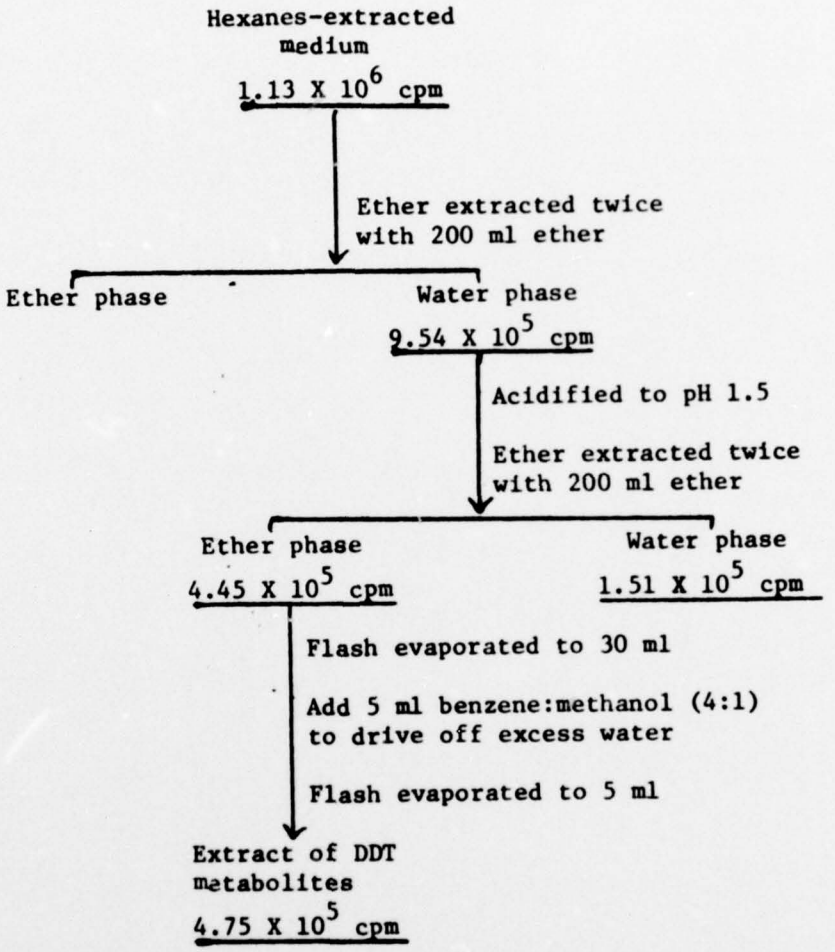
[e] Solvent was hexanes:ether:acetic acid (100:1:1).

using methanol: water (70:30) solvent, the non-esterified products gave 3 diffuse peaks with R_f values of approximately 0.27, 0.48, and 0.82. After methylation, only one peak at R_f 0.84 was observed. 2-Chlorosuccinate behaves similarly in that when unmethylated, its R_f value was 0.76, and the methylated derivative gave an R_f of 0.81. Thus, methylation may not greatly change the mobility of acids with this solvent system. It is tentatively assumed that all three diffuse peaks represent acids.

A second method of isolation of the DDT-degradation products has been developed and is now used. This procedure is outlined in Fig. 2, together with the recovery of radioactivity at each step. This procedure is less drastic than the first method since the drying and boiling steps are eliminated. The recovery in this instance was about the same as in the first procedure. The reason for the apparent loss of 50% of the radioactivity after extraction with acid is now being investigated. It should be noted, however, that the total extraction of ^{14}C from the medium was achieved after acidification, indicating that the metabolites are acids.

Purification of metabolites for GLC and mass spectroscopy. The final ether extract containing the DDT metabolites also contained nonradioactive products. These products interfered with the GLC analysis since their concentration was high enough to mask that of the DDT metabolites. Elimination of much of this interfering material was accomplished by TLC. This was achieved by placing 2.0 ml of the final ether extract on a TLC plate, which was developed in benzene:methanol (50:50). Most of the radioactivity remained at the origin, while the contaminating nonradioactive material moved and exhibited R_f values of 0.42 and 0.95. The ^{14}C-labeled material was removed

Figure 2. Extraction procedure for water-soluble products of DDT metabolism.



from the plate and eluted from the gel with water. The water was acidified and the products extracted into ether. Appropriate columns and operating conditions to allow for adequate separation of the volatile degradation products by GLC are currently being sought. This will then allow the metabolites to be injected directly, following gas chromatography, into the mass spectrometer for definitive identification.

Degradation products from marine isolates. Four of the marine isolates, all bacteria, most active in solubilizing DDT were grown in large volumes of a sea water medium. These cultures were extracted by the same procedures used for M. alternans. Although the extracts have not yet been characterized chemically, the water-soluble products are volatile at 55C at reduced pressure. Inasmuch as the same was true of the degradation products generated from DDT by M. alternans, it seems reasonable to believe that M. alternans is a suitable model for the marine bacteria.

Effect of various carbon sources on DDT degradation by natural marine communities. Addition of various carbon sources to natural microbial communities may enhance DDT metabolism in one of two ways. First, the carbon source may stimulate different groups of bacteria to become the predominant species in the community. Second, it may induce an enzyme system which, in addition to catalyzing a reaction involving its natural substrate, also acts to catalyze an initial phase in the degradation of DDT. Since the enzymes which cometabolize DDT are unknown, it is impossible to determine a priori what compound would stimulate the cometabolism of DDT. Consequently, nearly 100 different compounds, listed in Table 3, have been tested for their ability to enhance the conversion of DDT to water-soluble products.

Table 3. List of compounds added to natural marine communities in an attempt to enhance DDT solubilization.

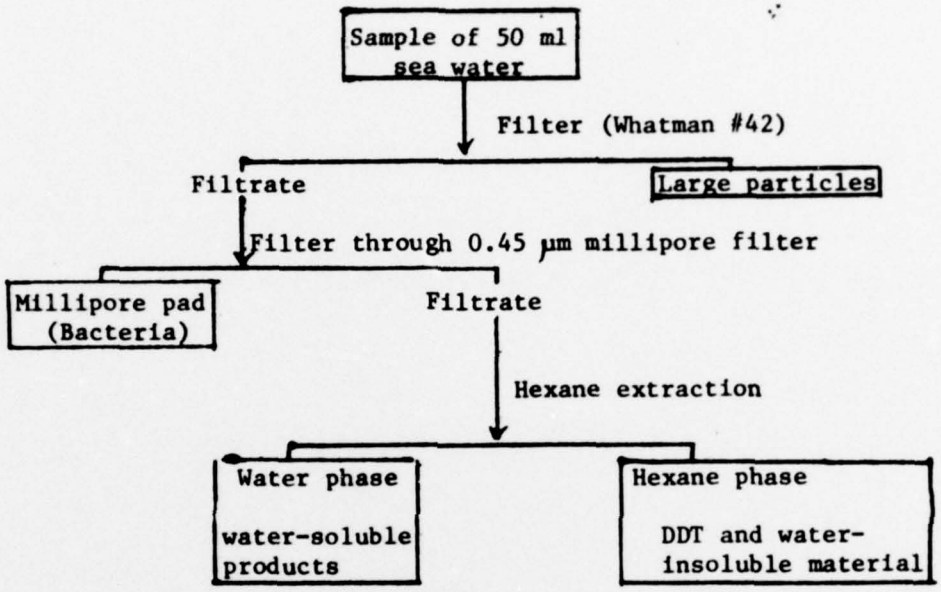
Acetic acid	Mannitol
Anthranilic acid	m-Methoxyphenol
4-Aminopyridine	Methylmalonic acid
m-Aminophenol	a-Naphtol, alpha
m-Aminobenzoic acid	Oleic acid
Alginate	2-Phenylbutyric
Ascorbic acid	α-Phenyl-o-cresol
4-Chlororesorcinol	4-Phenylbutyric acid
Coumalic acid	Pectin
Crotonic acid	Palmitic acid
Citrate	Phenylalanine
Catechol	p-Phenylphenol
Chitin	Phenylurea
Chlorodiphenylmethane	Phloroglucin
o-Cresol	Phthalic acid
Casamino acids	Picolinic acid
Dipyridyl (alpha, alpha)	Pyruvic acid
Diphenylmethane	Quinaldic acid
Ethoxybenzoic acid	Rutin
Gum arabic	Resorcinol
Glucose	Ribose
Guaiacol	Sebacic acid
Glutaric acid	Shikimic acid
Glycerol	Sodium lauryl sulfate
Gelatin	Starch
Glutamic acid	Succinate
p-Hydroxybenzoic acid	Syringic acid
4-Hydroxybutyric acid	Tannic acid
trans-β-Hydroxymuconic acid	Tartaric acid
4-Hydroxydiphenylmethane	p-Toluic acid
Kojic acid	2,4,6-Trihydroxybenzoic acid
Kynurenic acid	Tyrosine
Lauric acid	Urea
Lignin	Urethan
Linoleic acid	Vanillin
l-Leucine	Yeast extract
Maleic acid	
3-Methylcatechol	
4-Methylcatechol	
m-Methoxybenzoic acid	
o-Methoxybenzoic acid	
Mucic acid	

Since the Sagamore Terrace sampling site seemed to contain the most DDT decomposers, all the samples for this experiment were taken from that location. Each of the 100 Erlenmyer flasks (500 ml) received 250 ml of surface sea water. Course gravel from the upper 10 cm of sediment was placed in a clean container and mixed, and 50 ml of the course gravel was scooped into each of the 100 flasks. Test compounds (25 mg) were added to each flask. ^{14}C-labeled DDT was added 24 hr later at a concentration of 2 μg/ml and a specific activity of 1.0 μc/6.9 mg. Some of the flasks were incubated under anaerobiosis. Some were placed on a rotary shaker at 28 C, two were placed under continuous illumination, and the rest were incubated at 23 C in the dark. Initial samples were taken at 2 days and frozen at -30 C. Four samples were taken during the next 5 weeks and were also frozen after sampling. At the end of 5 weeks, the gravel was also removed and frozen. These samples will be extracted and the radioactivity of each fraction determined. The extraction and counting scheme to be used is outlined in Fig. 3.

Sorption of DDT by M. alternans. Mycelial mats of M. alternans were removed by filtration from the glucose-salts medium supplemented with ^{14}C-labeled DDT, and they were washed with sterile distilled water and returned to a fresh salts medium containing ^{14}C-DDT but lacking glucose. Counts of the radioactivity remaining in the medium were made during the incubation period. The results demonstrate that the mycelium bound most of the added DDT and then the bound material was slowly released (Table 4). Most of the released material was water-soluble, at least on the 9th day of incubation, since very little radioactivity was removed by hexane extraction.

To further characterize the pathway of metabolism, bacteria have

Figure 3. Scheme for extraction and determination of the distribution of 14-C-DDT in natural marine communities.



A box drawn around a fraction indicates that the radioactivity of that fraction will be measured.

Table 4. Sorption of DDT by Mucor alternans mycelium and the subsequent release of ^{14}C-labeled compounds.

Time after addition of DDT	Radioactivity in culture fluid (cpm/ml)
0	6484[a]
2 hr	687
1 day	3990
5 days	7288
9 days	6750

[a] Radioactivity in solution before mixing with fungus cells

been obtained which are able to grow on and degrade several analogues of DDT metabolites. The compounds are diphenylmethane, benzhydrol and phenylacetic acid, which are the non-halogenated analogues of bis-(p-chlorophenyl)methane (DDM), 4,4'-dichlorobenzhydrol (DBH) and p-chlorophenylacetic acid. Various of the isolates have been found to cometabolize DDM and DBH and to metabolize a variety of other compounds structurally related to likely intermediates in DDT biodegradation, but the products have not yet been identified.

C. DISCUSSION

Because of the persistence of DDT in marine and soil environments and its extensive use abroad, DDT residues continue to accumulate. Woodwell et al. (23) presented estimates of the accumulation of DDT in the oceans. The total amount was roughly 2.4×10^9 g, most apparently associated with marine algae (6,23). Sedimentation of organic matter, to which DDT is bound, transports residues to the abyss. DDT may accumulate in the abyss since these regions exhibit only limited bacterial degradative activity (12). In addition, the transfer rates for organic compounds out of the abyss are of the order of hundreds to thousands of years (23).

The lack of microbial degradation in nature is not the result of the nonexistence of species capable of destroying the insecticide because microorganisms able to degrade DDT extensively have been isolated from both soil and sewage (17,18). Moreover, marine isolates have been found that modify the two non-ring carbon atoms of DDT (16), but the production of ring-cleavage products owing to extensive enzymatic attack has not been demonstrated. We have used the solubilization of DDT as an index of extensive degradation of DDT; it must be acknowledged, however, that this criterion must be deemed an assumption only until these products are identified.

The results presented indicated that nearly 50% of the randomly chosen marine isolates solubilized 5% to 10% of the added DDT in 7 to 18 days. This is in agreement with data of Pfaender and Alexander (18) concerning the ability of sewage bacteria to degrade DDT. They found that as many as 90% of the randomly selected isolates from sewage to which glucose was added converted at least 10% of the DDT to products.

This poses the question: why is DDT not degraded quickly in the oceans or other bodies of water when so many microorganisms seem to be able to perform at least certain steps in the degradation.

A number of reasons for the persistence of compounds in nature have been proposed by Alexander (1). Some have a direct bearing on the persistence of DDT. One possibility is that the bacteria capable of degrading DDT are physically separated from it. Since the solubility of DDT in water is about 0.002 ppm and the solubility in animal fat is about 100,000 ppm (16), a large percentage of the DDT will be partitioned into fat. DDT is known to accumulate in algae (20,23), plankton (6), clams, oysters, fish, etc. Thus, the concentration of DDT in estuaries would be lowered by the entrance of DDT into living organisms. In addition, non-living organic material can bind DDT; for example, simple organic molecules (22) and dead algae (20). All or most of the DDT retained in animal fat would be unavailable for microbial degradation. The availability of DDT bound to non-living organic material is not presently known. We have demonstrated the sorption of DDT to M. alternans, this representing another example of the ability of microbial cells to bind DDT. Hicks and Corner (14) presented evidence that Bacillus megaterium retained a maximum of 1.7 µg of DDT/mg cell dry weight. About 75% of this DDT was located in the membrane. They also observed that the released material was more soluble than DDT. Microbial solubilization and possibly degradation may require binding of the insecticide to microbial cells, the result being that the species capable of DDT degradation are competing with all the other living and non-living substances that bind the pesticide.

Another reason for the persistence of DDT may be that microbial communities in the ocean inhibit or alter the ability of the active species

to decompose DDT. This hypothesis is supported by a report that a DDT-decomposing fungus was inhibited in its action on the pesticide by other soil fungi (4).

Low enzyme specificity or binding affinity, coupled with the very low concentration of DDT in water, could well be a major factor contributing to DDT persistence. Previous investigators have shown that DDT can be converted to 4,4'-dichlorodiphenylmethane (DDM) (11,17). If DDM is indeed produced in nature, removal of the chlorine atoms on the rings would produce diphenylmethane (DPM), which can be extensively metabolized by a number of bacteria (10). Nonspecific dehalogenases might be involved in this conversion; however, as far as is known, dehalogenases tend to be highly specific (13) and do not metabolize a wide range of substrates.

DDT is most likely cometabolized in nature since no one has yet isolated an organism able to grow on DDT as a sole source of carbon. Focht and Alexander (9) have shown that DDM can be converted to PCPA and that PCPA can be further metabolized by other microorganisms to ring fission products, possibly 3-chloro-substituted acids (8). Pfaender and Alexander have also demonstrated that PCPA can be further metabolized (17). Thus, enzyme systems do exist for the cometabolism of DDT, although no information concerning the specificity of these enzymes is known.

The presence of a p-chloro substituent on the rings of DDT presents a formidable obstacle to the ortho-1,2-oxygenases which commonly cleave aromatic rings. Dagley (7) presented evidence that ortho-cleaving enzymes were not generally active on rings with substituents on them. Ortho-cleavage would likely produce 2-chlorosuccinic acid; we have tested for

this compound, but none was found in culture fluids of M. alternans. The enzymes involved in meta-cleavage (i. e., catechol 2,3-oxygenase) are generally more active on compounds with substituents on the ring (7). A meta-type of ring cleavage would probably produce 3-chloro-substituted acids from DDT, as suggested by Focht and Alexander (8). In support of a meta-cleavage mechanism, Horvath (15) demonstrated that chloro-substituted catechols were metabolized by a meta-cleaving oxygenase. The production of 3-chloro-substituted acids from DDT seems to be plausible; hence, we plan to search for them in cultures of M. alternans and of the marine isolates.

The unknown water-soluble metabolites produced from DDT by M. alternans are probably acids. The products were shown not to be DDT, DDA, DBH, DBP, PCPA, or 2-chlorosuccinic acid. Thus, the water-soluble metabolites of DDT are possibly new breakdown products.

The hypothesis that nutrient availability is limiting the DDT-degrading species in marine waters is also being investigated. This hypothesis seems reasonable because the carbonaceous and nitrogenous nutrients source influence the ability of M. alternans to metabolize DDT (3).

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II. Section 2

A. MATERIALS AND METHODS

Media. Mucor alternans was grown in a glucose-salts medium containing 5 g glucose, 1 g NH_4NO_3, 1 g K_2HPO_4, 1 g KH_2PO_4, 0.2 g $MgSO_4$, 0.02 g $CaCl_2.2H_2O$, 0.01 g Fe_2SO_3, and 1000 ml distilled water (3). The basal sea water medium contained filtered, aged sea water containing 0.1% $(NH_4)_2SO_4$, 1% K_2HPO_4, and 1% K_2HPO_4. The growth factor stock solution contained 1 g yeast extract plus 1 g casamino acids per 100 ml of distilled water. Unless otherwise stated, growth factors were added to give a final concentration of 0.02%. Stock cultures were maintained on agar slants containing 1.0 g peptone, 2 g glucose, 0.05 g K_2HPO_4, 15 g agar, and 1000 ml of aged sea water.

Thin-layer chromatography. The chemicals were spotted on Chromatogram Sheet 6060 silica gel (Eastman) bearing a fluorescent indicator. After development, the spots were visualized with UVS 12 Mineralight (Ultraviolet Products, Inc., San Gabriel, Calif.). Chromatograms to be counted for their radioactivity were cut into 4 cm strips and counted with an Actigraph III strip counter (Nuclear-Chicago Corp.).

Gas-liquid chromatography. A Varian 1700 Gas Chromatograph equipped with a ^{63}Ni electron capture detector and two 6-ft X 1/8 in. glass colums was used. Pre-tested packing material was purchased from Applied Science Laboratories. The first column was 3% OV-1 on Gas-Chrom Q, AW, DMCS, 100/120 mesh. The other column was 10% DC 200 on Gas-Chrom Q, AW, DMCS, 100/120 mesh. Operating conditions were: 205 C, injector; 180 or 195 C, column; and 260 C, detector. The flow of N_2 gas through the column gave a retention time for DDT of about 20 min for the OV-1 column and 25 min for the DC 200 column.

Scintillation counting. Samples of 0.1 to 0.5 ml were placed into scintillation vials and 10 to 15 ml of Bray's scintillation fluid (7) was added. The vials were counted with a Mark I liquid scintillation counter (Nuclear Chicago Corp.).

Mass-spectrometry. Samples were adsorbed onto silica gel, and the gel was placed into a special capillary tube. Crystalline samples were directly added to the tubes. The samples were processed by the N.I.H. Biotechnology Resources Mass-Spectrometry Facility at the Dept. of Chemistry, Cornell University.

Nuclear magnetic resonance spectrometry. Samples of 10 to 30 mg were dissolved in 0.2 ml $CDCl_3$ (Mallinckrodt), and the spectra were obtained using a Varian 860A NMR Spectrometer.

Infra-red Spectrometry. Infra-red spectra were obtained on 1 to 5 mg samples incorporated into 100 mg KBr mini-discs using a Perkin-Elmer Infracord infra-red spectrophotometer.

Measurement of biological oxygen demand (BOD). BOD was measured in standard 300 ml bottles. The basal mineral salts medium contained 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4$, 0.025 g $CaSO_4.2H_2O$, 0.0025 g $FeSO_4.7H_2O$, 0.082 g $(NH_4)_2SO_4$, and 1 liter distilled water, and the pH was 7.0. The basal medium was aerated for at least 12 hr to saturate the medium with O_2. DDT analogs contained in an acetone solution were added to each bottle, the acetone was evaporated, and

then the oxygen-saturated basal medium was added with the inoculum. The bottles were incubated submerged in a water bath at 25 C. Dissolved oxygen was measured using a Biological Oxygen Monitor (Yellow Spring Instruments Co.). The alkali-azide modification of the standard iodometric method was used to standardize the oxygen meter for 100% saturation (2). After incubation and measurement of oxygen content, nitrite plus nitrate was measured by the method of Montgomery and Dymock (23).

Manometry. Standard manometric procedures were used (28). Each flask received 2 µmoles of substrate in acetone in the main compartment. The acetone was evaporated under a stream of N_2 gas, and 2.5 ml of sterile sea water was added to the dry substrate. The side arm received 0.5 ml of cells, and the center well received 0.2 ml of 20% KOH. Measurements were started by tipping in the cells from the side arm.

Chemicals. 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), 2,2-bis(p-chlorophenyl)acetic acid (DDA), 4,4'-dichlorobenzophenone (DBP), 4,4'-dichlorobenzhydrol (DBH), and p-chlorophenylacetic acid (PCPA) were obtained from Aldrich Chemical Co., Milwaukee, Wis. 2-Chlorosuccinic acid was purchased from K and K Laboratories, Plainview, N. Y. Labeled ^{14}C-DDT was provided by New England Nuclear, Boston, Mass. Hexanes used for extractions were pesticide grade from Fisher Scientific Co., Fairlawn, N. J. Column packing materials were purchased from Applied Science Laboratories, State College, Pa.

B. RESULTS

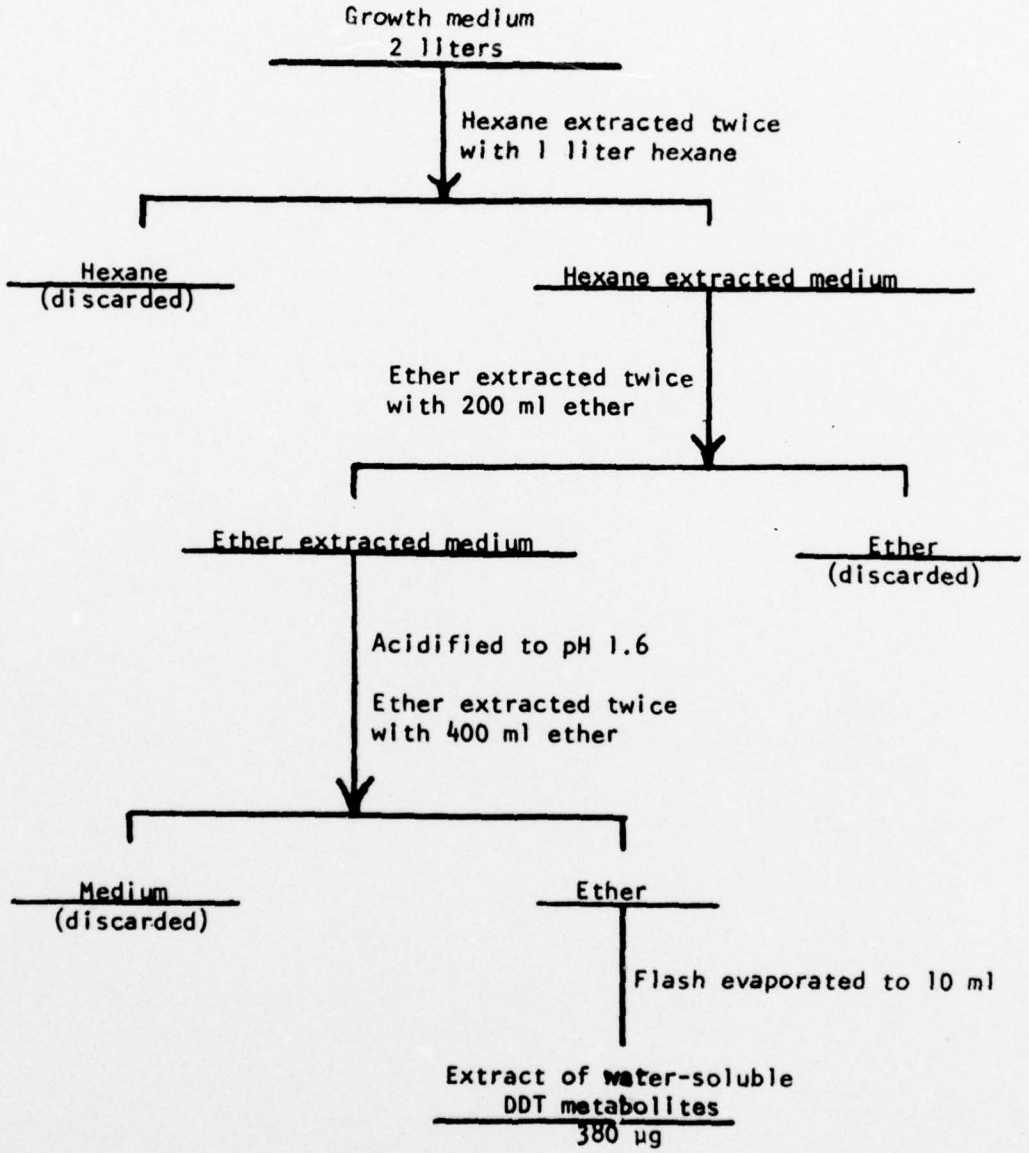
Water-soluble products formed by Mucor alternans. Mucor alternans converts 40% of the DDT supplied to the fungus to water-soluble products in 7 days (3). Since this rate of conversion was 3 to 4 times faster than that of the marine bacteria, the pathway of breakdown and the identities of the products formed by the fungus were investigated. Analysis of these metabolites by thin-layer chromatography and by gas-liquid chromatography has shown that the products may be unique and not described heretofore inasmuch as they were found to be distinct from 2,2-bis(p-chlorophenyl)acetic acid, 4,4'-dichlorobenzophenone, 4,4'-dichlorobenzhydrol, p-chlorophenylacetic acid, and 2-chlorosuccinic acid.

In order to obtain large amounts of the products, M. alternans was initially grown in a 10 liter fermentor in the presence of ^{14}C-labeled DDT. Under these conditions, however, M. alternans did not convert DDT to water-soluble metabolites. On the other hand, if the fungus was grown in 2 liter flasks containing 500 ml of broth plus ^{14}C-DDT and the products were pooled, nearly 800 µg of a preparation containing the products was obtained for each 4 liters of medium.

The water-soluble products were extracted with hexane and concentrated according the scheme shown in Figure 1.

Purification of water-soluble products. The water-soluble products in the ether extract were separated by thin-layer chromatography. A portion of the concentrated ether extract was applied in a narrow band on two silica gel plates and developed in methanol-water (75:25). After development, the plates were dried in air, and a strip was cut from the plate. The ^{14}C-containing areas on

Figure 1. Procedure for extracting water-soluble products of DDT metabolism.



the plate were located using an Actigraph III stripscanner. The results presented in Figure 2 indicate that at least three components were present, since three radioactive peaks were found. The procedure employed separated the radioactive compounds from the unlabeled yellow products. The dashed lines in Figure 2 designate the section of the chromatogram removed from the plates for extraction and further purification.

The silica gel containing the water-soluble products was extracted with water, and the liquid was filtered to remove the silica gel, acidified to pH 1.5, and extracted with ether. This ether extract was evaporated to 5 ml, the recovery of ^{14}C material being about 50%. The losses could not be accounted for, but presumably they occurred during flash evaporation. The entire extract was spread in a thin band on a second silica gel plate and developed in ethanol:-NH_4OH:water (140:7:28). The results of this separation are given in Figure 3. Some non-radioactive UV absorbing material was evident near the solvent front, but it was completely separated from the ^{14}C-labeled products. The two radioactive peaks designated S and F, were removed separately from the plate. Each was extracted with water, and the water phase was filtered, acidified, and extracted with ether. The recovery of ^{14}C material after concentration of the ether extract was 68%. At this stage of purification, about 24 µg of component S and 22 µg of component F remained.

Preparation of samples for mass spectrometry. One-half of extract S (12 µg) was placed in one small spot on a strip of silica gel and developed in methanol:-water (75:25). The strip was then examined for radioactivity. The result is shown in Figure 4. Two small faint UV-absorbing spots were observed, and these corresponded with the location of the ^{14}C activity on the gel. These spots and also a control portion of gel were removed and placed into small capillary tubes and analyzed by mass spectrometry. The mass spectrum of the control gel showed no peaks above m/e 57.

If these products contain chlorine, as do all reported products of DDT degradation, the parent ion and its fragments should show distinctive chlorine couplets by mass-spectral analysis. These couplets result from the natural chlorine isotopes, ^{35}Cl and ^{37}Cl, which occur in a 3:1 ratio. No such chlorine couplets were found for the two compounds in fraction S, and no parent ion was detected. Consequently, an identity or a molecular weight cannot yet be assigned to either component.

An insufficient quantity of product was present for a definitive mass spectrum. Consequently, the remaining 12 µg of fraction S was placed in one 50 mm^2 spot on a silica gel thin-layer plate; the gel within the spot was removed, placed into a capillary tube, and subjected to mass-spectral analysis. The resulting spectrum showed no distinctive Cl couplets and no parent ion could be identified. The major peaks were at m/e 177, 149, 147, 131, 129, 121, 105, 103, and 89.

Fraction F was analyzed by placing the entire extract (22µg) on one 80 mm^2 spot on a silica gel plate. A sample of the spot was placed into a capillary tube and examined by mass spectrometry. No parent peak or Cl couplets were found, however. The major m/e peaks were 146, 145, 119, 103, and 89.

Figure 2.

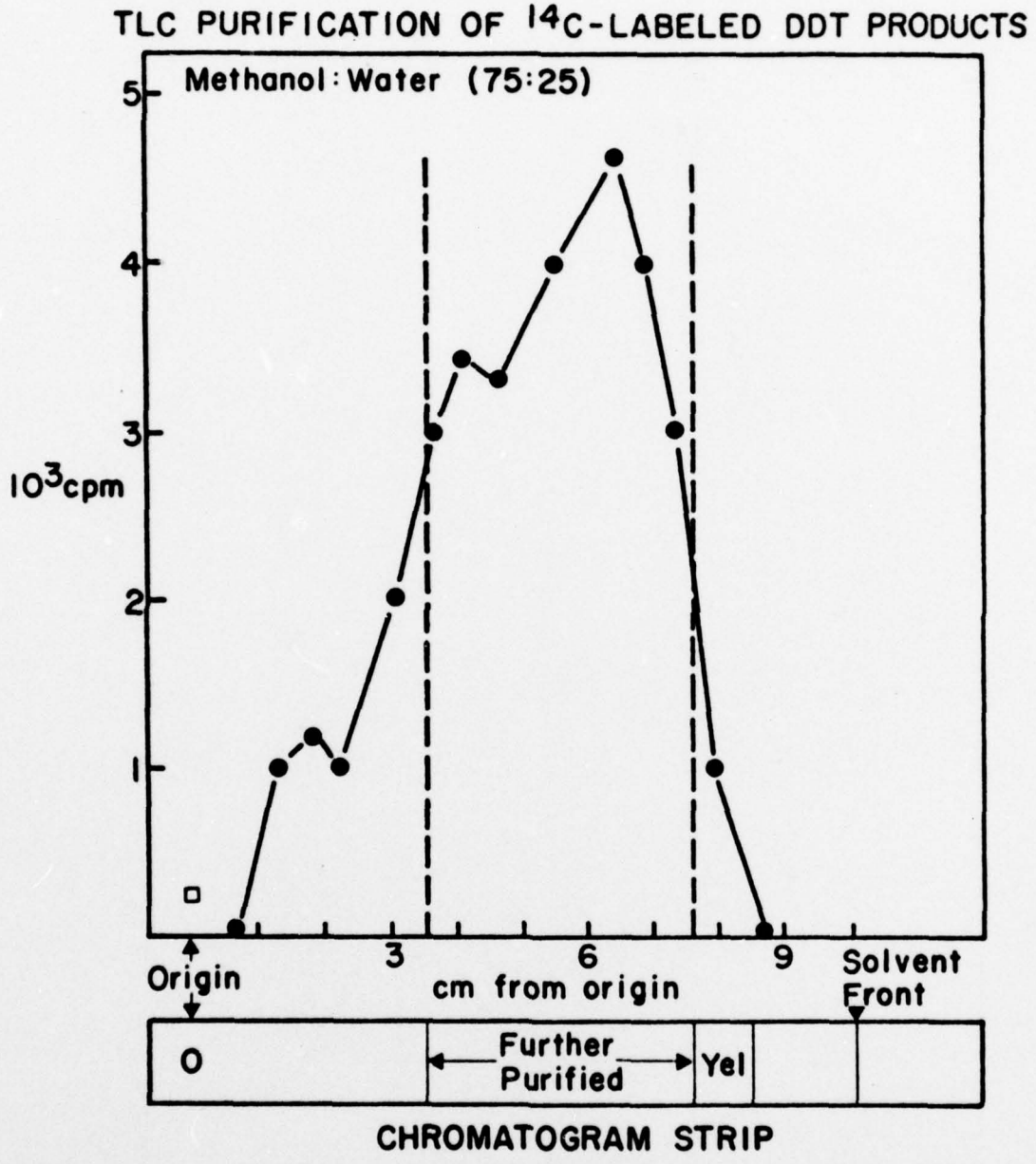

TLC PURIFICATION OF ^{14}C-LABELED DDT PRODUCTS
Methanol : Water (75:25)
5
4
3
2
1
10^3cpm
3
6
9
Origin
cm from origin
Solvent Front
0
Further Purified
Yel
CHROMATOGRAM STRIP

Figure 3.

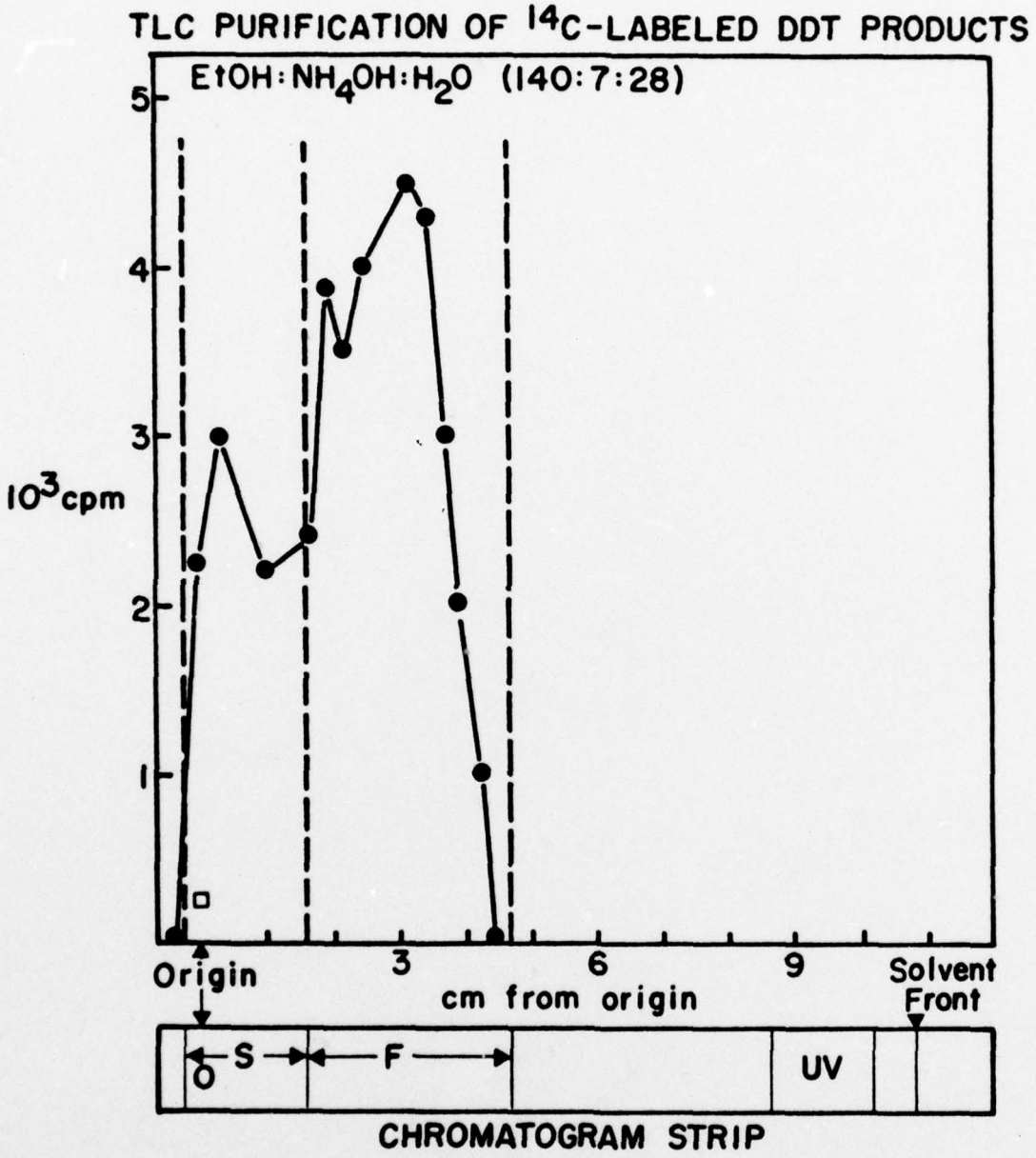
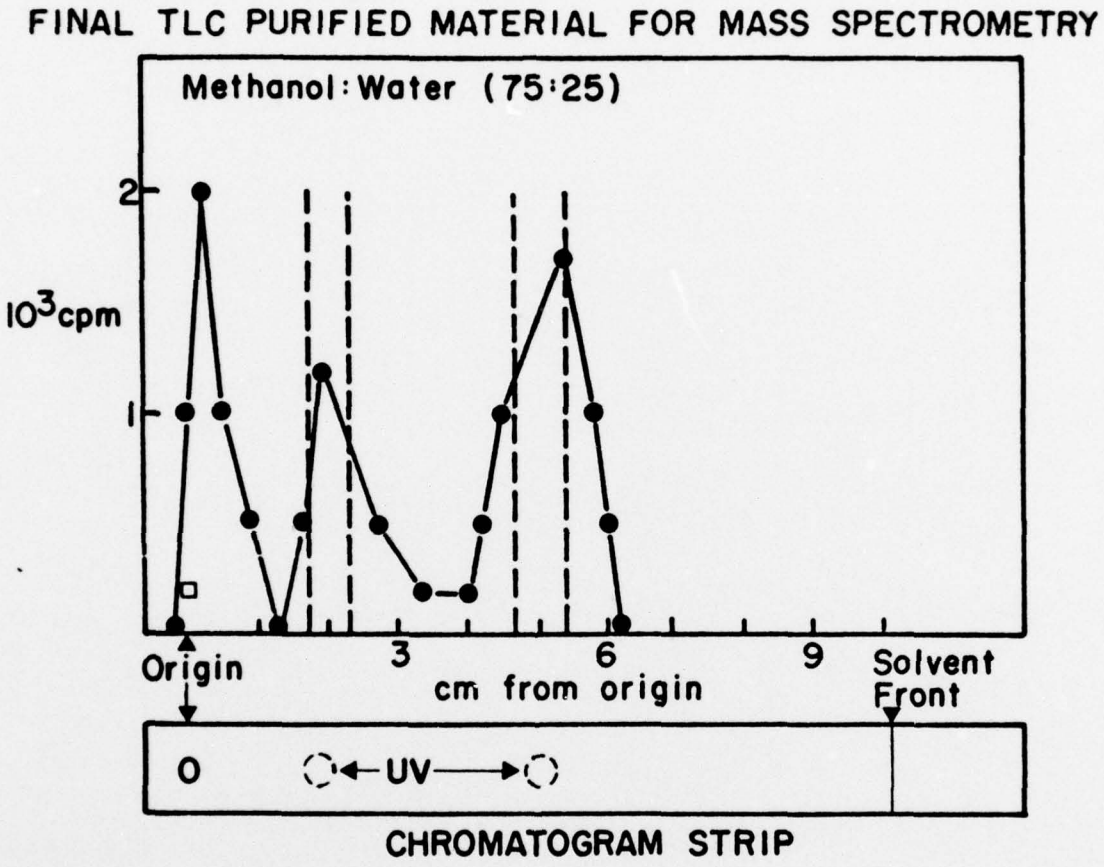

TLC PURIFICATION OF ^{14}C-LABELED DDT PRODUCTS
EtOH:NH4OH:H2O (140:7:28)
5
4
3
2
1
10^3cpm
Origin
3
6
9
Solvent Front
cm from origin
S
F
UV
CHROMATOGRAM STRIP

Figure 4.


FINAL TLC PURIFIED MATERIAL FOR MASS SPECTROMETRY
Methanol:Water (75:25)
2
1
10^3cpm
Origin
3
6
9
cm from origin
Solvent Front
0
UV
CHROMATOGRAM STRIP

Since the purity of components S and F was not established, the hypothesis that these water-soluble products do not contain chlorine must be tempered since impurities could have obscured the mass spectrum. On the other hand, these compounds may, in fact, not contain chlorine, particularly since they do not appear to be any of the DDT metabolites heretofore described.

Two approaches will be used to determine whether these metabolites contain chlorine. First, the release of chloride will be measured as M. alternans metabolizes DDT. If the products are halogen-free, the release of chloride should correspond stoichiometrically with the appearance of ^{14}C water-soluble products, the stoichiometric quantity being 5 moles of chloride per mole ^{14}C-labeled DDT. Second, the capacity of microorganisms from natural habitats to produce $^{14}CO_2$ from the labeled metabolites will be assessed: if these compounds do not contain para-substituted chlorine atoms, they presumably would serve as carbon and energy sources for many microorganisms. If the absence of chlorine is confirmed in subsequent studies, the water-soluble compounds are probably generated by a pathway involving the most extensive degradation of DDT so far reported for a single organism, since all products described to date contain chlorine. It should be pointed out, however, that several problems are associated with the identification of these metabolites; namely, extremely small amounts of material are available, rather large losses occur during each stage of purification, and crystalline chemicals have not yet been obtained.

Conversion of diphenylmethane to 1,1,1',1'-tetraphenyldimethyl ether. Because no microorganism has ever been found to grow at the expense of DDT, diphenylmethane was used as a carbon source to obtain species which might be able to alter DDT or to generate intermediates during the cometabolism of the insecticide. Bacteria were readily obtained using this non-chlorinated analogue of DDT as a carbon source, and two of the isolates were found to be able to solubilize DDT.

To further characterize how such molecules are destroyed, products formed during diphenylmethane metabolism were sought. One isolate was found to convert the non-halogenated analogue to 1,1,1',1'-tetraphenyldimethyl ether. This is the first report of the biosynthesis of this novel compound, and it represents a class of substances not previously sought, either in culture or in natural waters. Its identity was confirmed by mass spectral analysis and by nuclear magnetic resonance characteristics, and the bacterial product was confirmed to be the tetraphenyl compound by a comparison of its melting point, solubility characteristics, and infra-red spectrum with those of an authentic preparation synthesized by the method of Pratt and Draper (27). Such a dimerization to yield the ether is unique in the biological alteration of insecticides.

Further work is currently in progress to establish the compounds synthesized as these bacteria cometabolize other intermediates known to be excreted as DDT is transformed in nature or in culture; e.g., 4,4'-dichlorodiphenylmethane, 4,4'-dichlorobenzophenone (DBP), and 4,4'-dichlorobenzhydrol (DBH).

Effect of organic matter on DDT degradation by marine communities. The addition of organic matter to natural microbial communities may enhance DDT metabolism because: (a) various carbon sources may stimulate dissimilar groups of bacteria to become the predominant species in the community or (b) one or more may induce an enzyme system which, in addition to catalyzing a reaction involving the natural substrate, also catalyzes an initial phase in the degradation

of DDT. Since the enzymes which cometabolize DDT are unknown, it is impossible to determine a priori what compound would stimulate the cometabolism of DDT. Consequently, nearly 80 compounds listed in Table 1 were tested for their ability to enhance the conversion of DDT to water-soluble products.

Since the Sagamore Terrace site contained an abundance of DDT-metabolizing bacteria (18), all the samples for this experiment were taken from that location. Each of the Erlenmeyer flasks (500 ml) received 250 ml of surface sea water. Course gravel from the upper 10 cm of sediment was placed in a clean container and mixed, and 50 ml of the course gravel was scooped into each of the flasks. Test compounds (25 mg) were added to each reaction vessel. Carbon-14-labeled DDT was added 24 hr later at a concentration of 2 µg/ml and a specific activity of 1.0 µc/6.9 mg. Some of the flasks were incubated under anaerobiosis, some were placed on a rotary shaker at 28 C, two were placed under continuous illumination, and the rest were incubated at 23 C in the dark. Initial samples were taken at 2 days and frozen at -30 C, and 4 samples were taken during the next 5 weeks and were also frozen. At the end of 5 weeks, the gravel was removed and frozen. The water-soluble products were extracted according the scheme shown in Figure 5, and the radioactivity was then measured.

The 5-week samples were analyzed first for production of water-soluble products. The results are shown in Tables 1-3. It is apparent from these data that in no case were water-soluble DDT metabolites produced by these model ecosystems. The average recovery of ^{14}C added material was approximately 90%. The few very low and very high recoveries probably resulted from the particulate nature of DDT in solution (6), as indicated by its lack of passage through a Whatman no. 42 filter paper; i.e., DDT was not distributed uniformly in each sample taken from the 500 ml flask. For the few samples with ^{14}C remaining in the filtrate, all was removed by Millipore filtration or by hexane extraction.

These results using model marine communities are in marked contrast with the results of studies of pure cultures of marine bacteria, in which it was observed that nearly 50% of the individual bacteria were capable of solubilizing DDT. In the present study, none of the natural communities produced such metabolites.

Metabolism of para-substituted aromatic compounds. In order to degrade DDT extensively, a microorganism must have the ability to remove the chlorine atoms from the aromatic rings. It has been shown that adenosine deaminase, which catalyzes the removal of amino groups from adenosine, can also remove a chlorine substituted in place of the amino group. Since other enzymes removing carbon-linked substituents may exhibit a similar capacity to effect a dechlorination reaction, a number of marine bacteria able to grow on para-substituted aromatic compounds were sought.

To isolate bacteria capable of using individual aromatic compounds as sole sources of carbon, enrichment cultures were established. Each enrichment contained 50 ml of sea water basal broth plus 25 mg of the aromatic compound. Each was inoculated with 1.0 ml of surface water plus sediment from the Sagamore Terrace site. After 5, 9, and 30 days of incubation, approximately 0.05 ml of the enrichment was spread on agar plates containing 0.025% of the appropriate aromatic compound. Colonies appearing on these plates were picked and inoculated into a basal broth medium containing 0.025% of the same aromatic compound plus growth factors. Those isolates able to grow in the broth were isolated and stored on slants.

Table 1. Effect of nutrient additions on the ability of model marine ecosystems to convert ^{14}C-DDT to water-soluble products.

Carbon source	CPM before filtration	CPM after filtration	% ^{14}C recovered on filter pad
Acetic acid	144,000	0	93
Anthranilic acid	112,000	0	102
4-Aminopyridine	70,800	0	169
m-Aminophenol	144,000	0	74
m-Aminobenzoic acid	71,100	0	74
Alginate	95,900	0	91
Ascorbic acid	105,000	0	97
4-Chlororesorcinol	119,000	0	-
Coumalic acid	123,000	0	89
Crotonic acid	169,000	0	60
Citrate	135,000	0	92
Catechol	73,000	0	125
Chitin	103,000	600	91
Chlorodiphenylmethane	114,000	1073	82
o-Cresol	192,000	0	78
Casamino acids	141,000	0	84
Dipyridyl (alpha,alpha)	198,000	0	73
Diphenylmethane	153,000	0	-
Ethoxybenzoic acid	189,000	0	77
Gum arabic	80,400	0	86
Glucose	153,000	0	93
Guaiacol	143,000	0	92
Glutaric acid	148,000	0	102
Glycerol	171,000	0	67
Gelatin	118,000	0	91
Glutamic acid	140,000	580	103
p-Hydroxbenzoic acid	159,000	0	81
4-Hydroxybutyric acid	153,000	0	82
trans-β-Hydroxymuconic acid	150,000	0	103
4-Hydroxydiphenylmethane	98,900	0	116
Kojic acid	220,000	0	57
Kynurenic acid	166,000	0	90
Lauric acid	89,000	0	117
Lignin	120,000	0	83
Linoleic acid	150,000	0	81
L-Leucine	105,000	0	128
Maleic acid			
3-Methylcatechol	154,000	0	105
4-Methylcatechol	127,000	536	81
m-Methoxybenzoic acid	220,000	0	65
o-Methoxybenzoic acid	88,000	0	72
Mucic acid	199,000	0	77

Table 1 - continued

Mannitol	131,000	487	64
m-Methoxyphenol	140,000	0	99
Methylmalonic acid	141,000	0	97
Naphthol, alpha	134,000	0	85
Oleic acid	98,000	0	106
2-Phenylbutyric acid	129,000	0	88
α-Phenyl-o-cresol	149,000	0	84
4-Phenylbutyric acid	105,000	659	95
Pectin	179,000	0	85
Palmitic acid	102,000	0	99
Phenylalanine	92,600	0	72
p-Phenylphenol	139,000	0	94
Phenylurea	117,000	0	104
Phloroglucinol	124,000	0	96
Phthalic acid	122,000	0	93
Picolinic acid	72,400	0	83
Pyruvic acid	151,000	0	90
Quinaldic acid	110,000	0	112
Rutin	116,000	0	89
Resorcinol	130,000	0	90
Ribose	183,000	0	73
Sebacic acid	120,000	0	120
Shikimic acid	104,000	0	88
Sodium laurylsulfate	119,000	0	-
Starch	104,000	0	77
Succinate	260,000	0	58
Syringic acid	121,000	0	92
Tannic acid	321,000	0	53
Tartaric acid	92,300	0	82
p-Toluic acid	95,000	0	78
2,4,6-Trihydroxybenzoic acid	236,000	0	56
Tyrosine	133,000	652	73
Urea	147,000	536	78
Urethan	215,000	0	63
Vanillin	152,000	0	80
Yeast extract	112,000	0	88

Figure 5. Scheme for extraction and determination of the distribution of ^{14}C-DDT in natural marine communities.

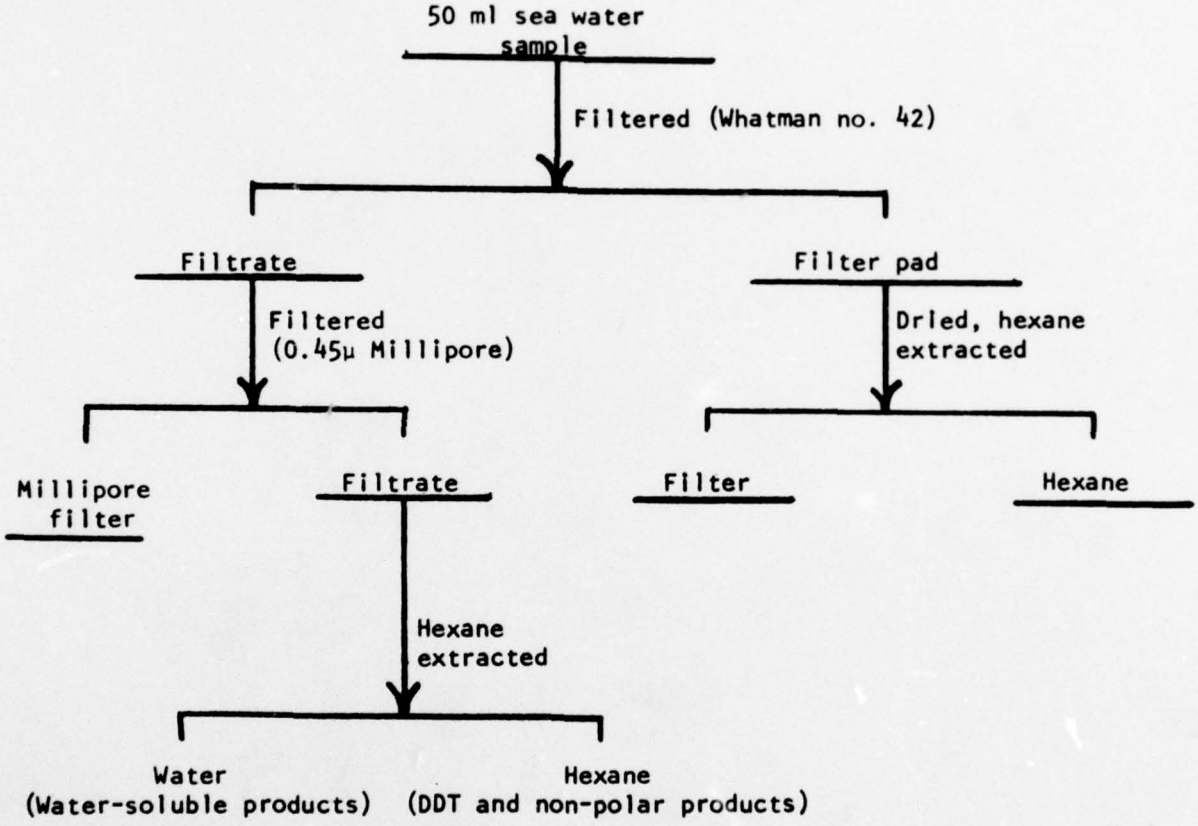


Table 2. Effect of nutrient additions on the ability of model marine ecosystems to convert DDT to water-soluble products under anaerobic conditions.

Treatment	Nutrient addition	CPM before filtration	CPM after filtration	% ^{14}C recovered from filter pad
Dark	No additions	15,200	0	109
Dark	+N+P	40,900	701	44
Dark	+N+P+Glucose	42,500	8533	43
Dark	+N+P+Catechol	23,500	8378	55
Dark	+N+P+Rutin	42,600	0	30
Dark	+N+P+Palmitic acid	44,000	0	57
Dark	+N+P+Diphenylmethane	33,400	2192	51
Dark	+N+P+4-Methylcatechol	52,700	0	84
Dark	+N+P+4-Phenylbutyric acid	42,100	2149	43
Light	No additions	22,000	0	90
Light	+N+P	29,600	1157	96

Table 3. Effects of nutrient additions on the ability of model marine ecosystems to convert DDT to water-soluble products under aerobic conditions.

Treatment	Nutrient addition	CPM before filtration	CPM after filtration	% ^{14}C recovered from filter pad
Dark	No additions	43,900	0	138
Dark	+N+P	76,900	0	108
Dark	+N+P+Catechol	83,200	0	106
Dark	+N+P+Palmitic acid	84,400	0	113
Dark	+N+P+Diphenylmethane	75,200	443	93
Dark	+N+P+4-Methylcatechol	68,400	0	25
Dark	+N+P+4-Phenylbutyric acid	152,000	548	87

After approximately 6 weeks of incubation, 1.0 ml of the primary enrichment cultures was transferred to fresh basal sea-water medium containing 0.025% of the aromatic compound but no growth factors. Additional isolates were obtained and stored on slants.

A third enrichment was started using an inoculum consisting of 1.0 ml of the second enrichment culture, which had been incubated for about 8 weeks. The third enrichment solution contained the basal medium, 0.1 ml of a growth factor solution, and 0.5% of the aromatic compound. Turbidity developed in cultures containing p-aminobenzoic acid, p-anisic acid, and p-methoxyphenylacetic acid. Additional isolates were obtained from enrichment cultures containing these three aromatics.

The results of these enrichments and the number of isolates which grew in liquid media containing each aromatic chemical are given in Table 4. Many of the isolates proved to be agar digesters or failed to grow in the liquid media.

Responses of isolates to concentration of aromatic compounds. Since the aromatics tested might have been toxic, the ability of the isolates to grow at various concentrations of the chemicals was determined. Isolates were grown in 10 ml of basal broth containing 0, 5, 10, and 30 mg of the aromatic compound which was used for their isolation. Some of the bacteria were unaffected by the presence of the aromatic, several were inhibited at all concentrations, and a few were stimulated by one concentration but inhibited or unaffected by another. Three classes of response to aromatics compounds are presented in Table 5. All of the bacteria grew to some extent on the growth factors in the medium, even if the solution had no aromatic compound. The isolate growing in the presence of p-toluic acid was not stimulated by low concentrations (0.05% and 0.10%) while 0.30% inhibited its growth. The growth of the p-aminophenylacetate-utilizer was about the same at 0, 0.05 and 0.10%, but it was markedly stimulated at 0.30%. The p-anisic acid-utilizing bacterium was stimulated at 0.05% and 0.10% but nearly completely inhibited at 0.30%. None of these microorganisms grew in the absence of growth factors.

Since the ability to oxidize para-substituted aromatics may indicate the enzymatic capacity to remove the para-substituent, the potential metabolism of DDT analogs, known DDT metabolic products, and a few related chemicals by nine of the marine bacteria will be studied. Isolates particularly active in oxidizing chlorine-substituted molecules will then be tested for dechlorinase activity by direct measurement of chloride release. If metabolic products accumulate and can be crystallized, their identity will be determined.

Oxidation of compounds related to DDT by a marine bacterium. An isolate obtained from enrichments containing p-anisic acid was tested for its ability to oxidize compounds related to DDT. Standard manometric procedures were used (28). The bacterium was grown in two 500-ml flasks, each containing 250 ml of basal sea water medium supplemented with 1% glucose and growth factors but no p-anisic acid. The cells were removed from the growth medium by centrifugation and washed twice with sterile sea water. The final cell suspension in sea water contained 3.2×10^{11} cells per ml, and each Warburg flask received a 0.5 ml portion. Two micromoles of each potential substrate listed in Table 6 was added in an acetone solution since most were not sufficiently water soluble. The ace-

Table 4. Results of enrichment culture for isolation of microorganisms able to use para-substituted aromatics as sole sources of carbon and energy.

Class of compounds	Substituent in para- position	Actual compound used	Total no. of isolates	Positive growth response
Phenols	$-NH_2$	p-Aminophenol	3	0
	$-NO_2$	p-Nitrophenol	5	0
	$-CH_3$	p-Cresol	N	N
	$-OCH_3$	p-Methoxyphenol	4	0
Benzoic acids	$-NH_2$	p-Aminobenzoic acid	1	1*
	$-NO_2$	p-Nitrobenzoic acid	1	0
	$-CH_3$	p-Toluic acid	2	1
	$-OCH_3$	p-Anisic acid	1	1*
Toluenes	$-NH_2$	p-Toluene	2	0
	$-NO_2$	p-Nitrotoluidine	3	0
	$-CH_3$	p-Xylene	4	0
	$-OCH_3$	p-Methylanisole	2	1
Biphenyls	$-NH_2$	Benzidine	3	0
	$-NO_2$	4,4'-Dinitrobiphenyl	7	1
	$-CH_3$	p,p'-Bitolyl	8	0
	$-OCH_3$	4,4'-Dimethoxybiphenyl	7	1
Phenylacetic acids	$-NH_2$	p-Aminophenylacetic acid	6	2*
	$-NO_2$	p-Nitrophenylacetic acid	1	0
	$-CH_3$	p-Tolylacetic acid	0	0
	$-OCH_3$	p-Methoxyphenylacetic acid	2	1

N = Not tested.
* = Chosen for further investigation.

Table 5. Typical growth responses of bacteria to various concentrations of aromatic compounds

Conc. (%)	OD_{550} after 300 hr incubation		
	p-Toluic acid utilizer	p-Anisic acid utilizer	p-Aminophenylacetic acid utilizer
0	0.14	0.13	0.14
0.05	0.15	0.22	0.17
0.10	0.17	0.37	0.14
0.30	0.08	0.02	0.37

tone was evaporated under a stream of nitrogen gas, and sea water was added to the residual dry chemical. The compounds tested were: (a) DDT; (b) known metabolic products of DDT---DDD, DDE, DDA, DBP, DBH, DDM, and PCPA; and (c) DDT analogs and structurally related molecules---methoxychlor, bis(4-hydroxyphenyl)methane, 1,1-diphenyl-2,2,2-trichloroethane, 4-hydroxydiphenylmethane, p-hydroxybenzophenone, benzophenone, 4-chlorobenzophenone, benzhydrol, 4-chlorobenzhydrol, 4-chlorobensoic acid, p-aminophenylacetic acid, p-aminobenzoic acid, p-methoxybenzoic acid, o, p-methoxyphenylacetic acid, and phenylacetic acid.

Some of the results of this preliminary study are presented in Tables 6 and 7 Endogenous O_2 uptake has been substracted from all values presented. The results indicate that this bacterium could oxidize p-chlorophenylacetic acid and phenylacetic acid, and some of the other compounds may have been oxidized as well. In future studies, the cells will be grown in the presence of the para-substituted aromatic to insure induction of requisite enzyme systems and O_2 uptake will be measured over a longer period of time.

Relation of chemical structure to biodegradability. The use of biological oxygen demand as a measure of biodegradability offers one major advantage to manometric techniques: long incubation periods are practical. Indeed, prolonged incubation may be necessary to measure degradation of recalcitrant molecules. Reliable results can be obtained providing the inoculum is small and the amount of substrate to be tested is adjusted to such a level that complete oxidation results in nearly total depletion of the dissolved O_2 in the bottle. Nitrate and nitrite levels in all bottles showing O_2 consumption were measured to show that O_2 depletion was not due to nitrification. In these studies, the inoculum consisted of 1.0 ml of a 1:100 dilution of soil. The BOD bottles contained about 7.6 mg dissolved O_2. At regular time intervals, the O_2 level in selected bottles was measured with an O_2 meter, and the bottle was then discarded. Each point in the figures represents the data from a single BOD bottle.

The results obtained with various diphenylmethanes are depicted in Figure 6. Unsubstituted diphenylmethane or p-hydroxydiphenylmethane resulted in nearly complete depletion of O_2 from the BOD bottles. When both rings contained para-substituted nitro, chloro, or even hydroxyl groups, however, little or no degradation occurred.

Similar results were obtained with various benzophenones. As shown in Figure 7, benzophenone and the two mono-substituted benzophenones tested were biodegradable. On the other hand, little O_2 was consumed if both rings had substituents at the para positions; that is, they were resistant to biodegradation under the test conditions.

When phenylacetic acid was the substrate in the BOD bottles, essentially all of the dissolved O_2 was consumed in 2 days. However, no biodegradation was evident when several other para-substituted compounds were tested by the BOD technique (Figure 8). In addition, unsubstituted diphenylbenzhydrol appeared resistant to biodegradation as assay by the BOD system. On the other hand, microorganisms able to grow on this chemical have been obtained. These studies indicate that para-substituents on aromatic rings increase resistance of the chemicals to microbial attack, with para-substituted chlorine being one of the more effective means of slowing microbial attack.

Table 6. Ability of isolate 8b to oxidize para-substituted aromatic compounds.

Minutes	O_2 consumed, µmole				
	p-Methoxy-benzoic acid	p-Aminophenyl-acetic acid	p-Methoxyphenyl-acetic acid	p-Hydroxy-benzophenone	Phenyl-acetic acid
30	0.2	0.0	0.1	0.0	1.7
60	0.0	0.0	0.2	0.5	2.4
90	0.6	0.1	0.3	0.7	2.6
120	0.8	0.1	0.8	1.0	3.3
145	1.1	0.3	0.8	1.4	3.4
Calc*	17	18	20	29	18

*Quantity of O_2 for complete oxidation of 2 µmoles of substrate.

Table 7. Ability of isolate 8b to oxidize *para*-substituted aromatic compounds

	O_2 consumed, µmole				
Minutes	DDT	DDA	PCPA	Methoxychlor	*p*-Aminobenzoic acid
30	0	0.2	3.3	0.4	0.0
60	0.2	0.4	5.0	0.7	0.0
90	0.3	0.5	5.6	0.8	0.1
120	0.7	0.8	6.1	1.3	0.1
145	0.7	0.9	6.6	1.3	0.3
Calc*	30	30	17	36	14

*Quantity of O_2 for complete oxidation of 2 µmoles of substrate.

Figure 6.

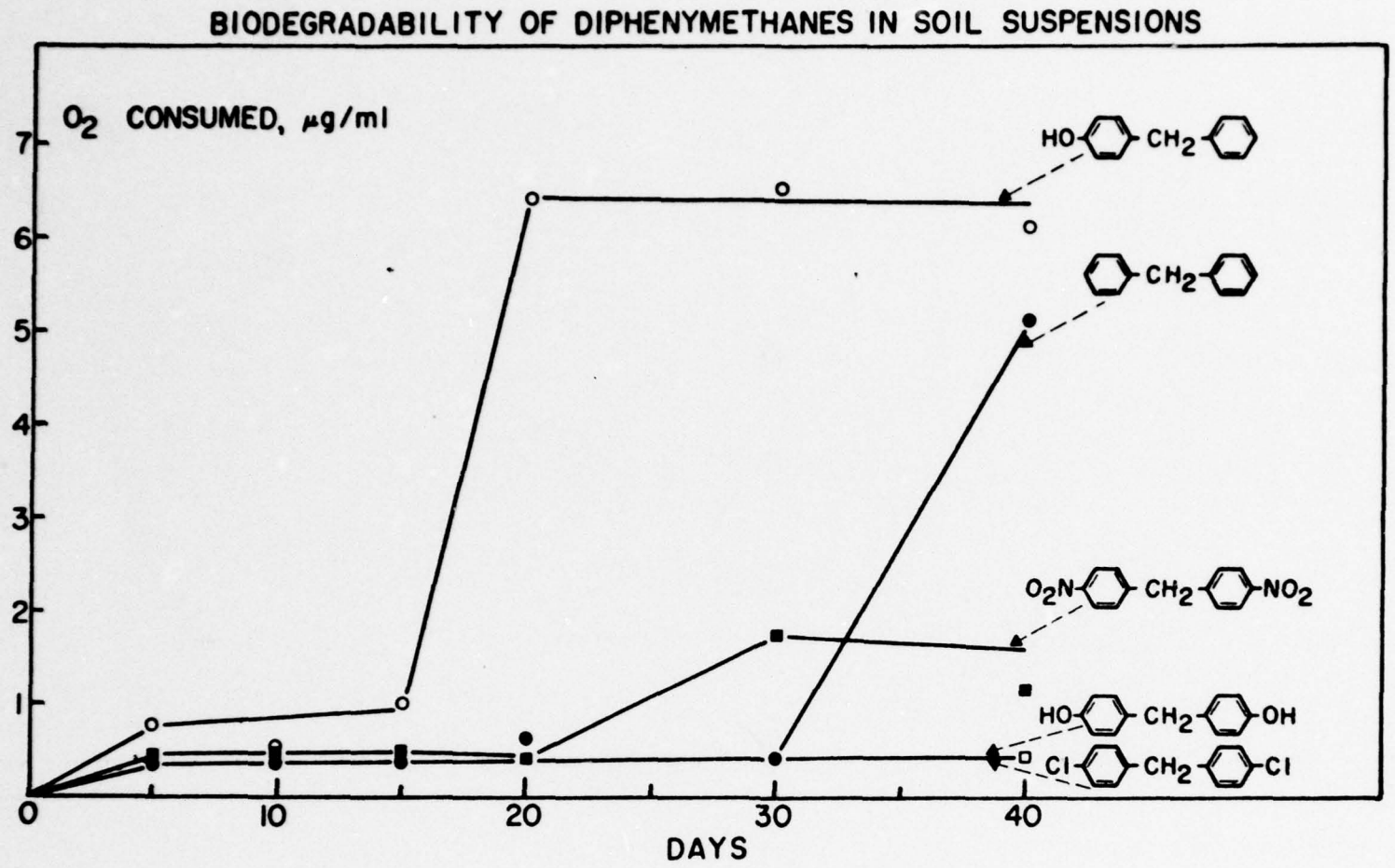

BIODEGRADABILITY OF DIPHENYMETHANES IN SOIL SUSPENSIONS
O_2 CONSUMED, µg/ml
7
6
5
4
3
2
1
0
10
20
30
40
DAYS
HO CH2
CH2
O2N CH2 NO2
HO CH2 OH
Cl CH2 Cl

Figure 7.

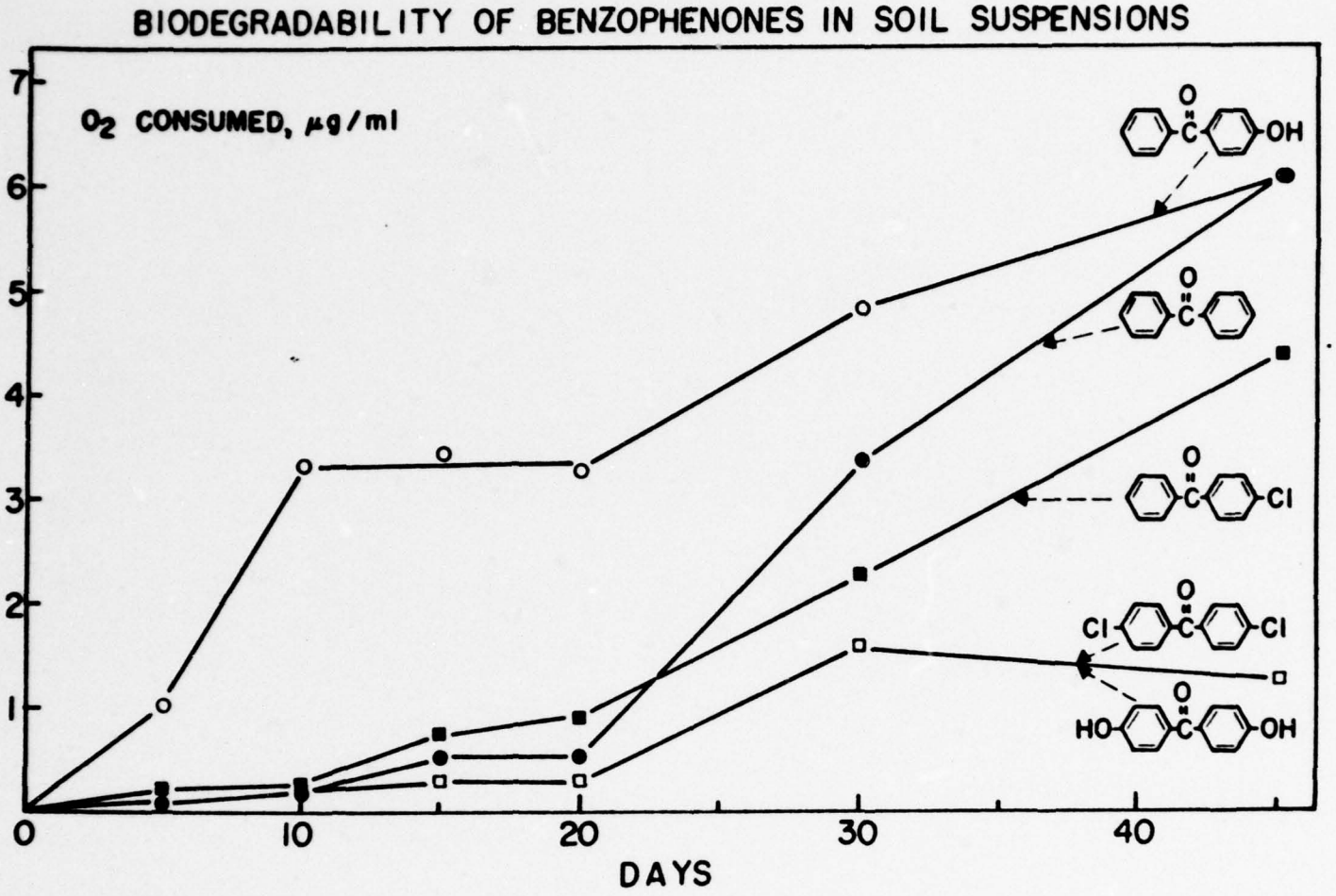
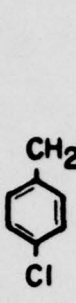
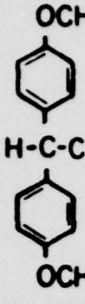
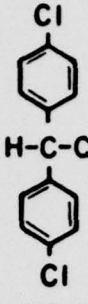
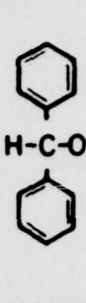
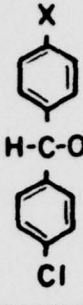

BIODEGRADABILITY OF BENZOPHENONES IN SOIL SUSPENSIONS
O_2 CONSUMED, µg/ml
7
6
5
4
3
2
1
0
10
20
30
40
DAYS
OH
Cl
Cl
Cl
HO
OH

Figure 8.

BOD OF DDT AND RELATED COMPOUNDS

DAYS	OXYGEN CONSUMED, µg/ml				
					
2	0.4	0.7	0.4	–	–
5	0.4	0.7	0.4	0.2	0.1
15	0.2	0.5	0.4	0.1	0.2
30	1.4	1.4	0.7	0.9	0.5
40	3.	1.1	0.4	1.0	0.4

X = H or Cl

Factors affecting DDT biodegradation in marine waters. The environmental factors now being tested for their influence on DDT degradation are: (a) salinity---1.015, 1.022, and 1.035; (b) temperatures of 4°, 15°, 23°, and 32° C; (c) the addition of carbon sources to surface waters---25 mg of algal cells (lyophilized Cylindrospermum), 1.0 ml of raw sewage, and 0.02 ml DPM; (d) the same additions to a surface water-mud model ecosystem; (e) the same additions to a surface water-sand model ecosystem; (f) aerobic conditions; (g) anaerobic conditions; and (h) water-saturated marine sediments---gravelly coarse sand, fine sand and fine sandy loam. Six 125 ml bottles are used for every variable, and each bottle contains 75 ml of sea water or sea water plus sediment. The diphenylmethane was added either at the start of the incubation or at the start and again at 2, 5, 8, and 12 weeks. Information on the effects of these additions on the ability of microorganisms to metabolize DDT may enable more meaningful prediction of the behavior of DDT in various marine situations.

DDT was added to each bottle to a final concentration of 50 µg/ml. The incubation was at 23 C in the dark, unless otherwise stated. At 0, 2, 5, 8, 12, and 27 weeks, one of the six bottles for each variable was removed and placed in the freezer for later analysis. The procedure for extraction and concentration of these samples is summarized in Figure 9. The extracted samples are currently being analyzed by gas-liquid chromatography on 3% OV-1 and 10% DC200. Preliminary determinations made of the 12-week samples indicate that little or no transformation of DDT occurred in some of the test conditions while rather extensive alteration was apparent in others.

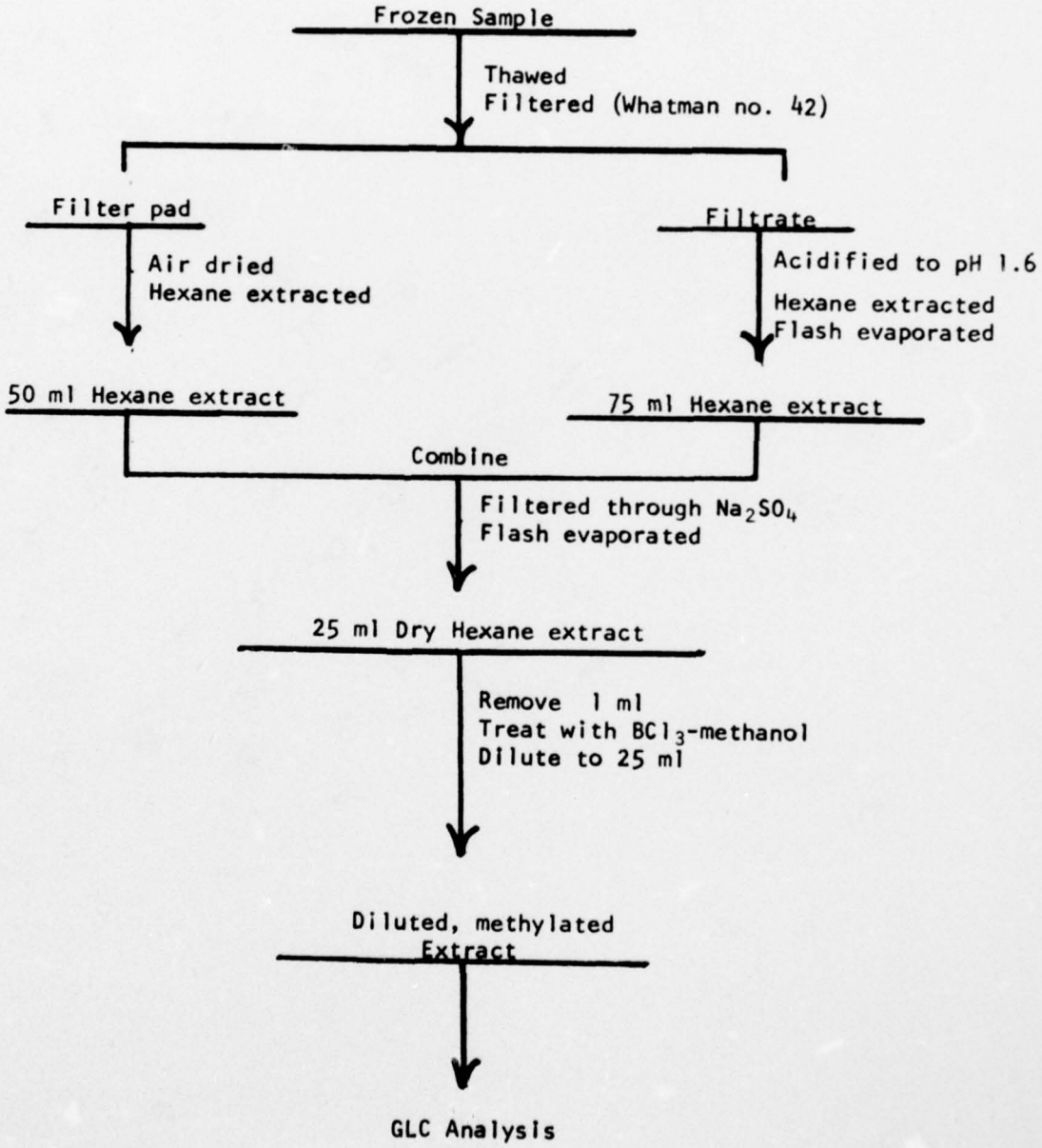
C. DISCUSSION

A wealth of data documenting the conversion of DDT to DDD and DDE exists in the published literature. These products have been found in soil, water, plants, and animals. Although these compounds can be generated by photochemical reactions (21, 26), most of it seems to be the result of biological action, principally that of microorganisms. In addition to the transformation to DDD and DDE, other and more extensive alterations of DDT have been reported. Such DDT metabolites as 1-chloro-2,2-bis(p-chlorophenyl)ethylene (DDMU), unsym-2,2-bis(p-chlorophenyl)ethylene (DDNU), 1-chloro-2,2-bis(p-chlorophenyl)ethane (DDMS), 2,2-bis(p-chlorophenyl)methane (DDM), 4,4'-dichlorobenzophenone (DBP), 4,4'-dichlorobenzhydrol (DBH), 2,2-bis(p-chlorophenyl)acetic acid (DDA), and p-chlorophenylacetic acid (PCPA) are generated in model freshwater and sewage ecosystems under controlled laboratory conditions (25). Most of these metabolites have not been isolated from marine environments, but this may result from the minute amounts of the intermediates accumulating in nature.

Except for a report that DDM can be transformed directly to PCPA by microorganisms (14), no evidence for ring cleavage products from DDT has been reported. Recently two independent research groups isolated and identified bis(p-chlorophenyl)acetonitrile from sewage amended with DDT (1, 17). The fate of this compound in nature and its toxicity have not been described.

DDT and DDE have been detected and reported to accumulate in marine environments along with DDT (9). Whether these compounds were generated by the activity of microorganisms or by photochemical reactions is not known. However,

Figure 9. Scheme for extraction and preparation of samples for analysis by gas-liquid chromatography.



Patile et al. (24) have shown that marine microorganisms have the ability to convert DDT to such products. Some of their isolates were noted to synthesize DDOH and DDNS as well as DDD and DDE. Products of more extensive degradation have not been reported in marine waters.

Considering the large literature on DDT, its persistence in nature, and its concentration through food chains, it is surprising that the fate of only one of the 14 carbon atoms in the molecule is known. This may partly result from the widespread use of hexane extraction procedures, and consequently only hexane-soluble molecules are characterized. By contrast, Anderson et al. (3) isolated a fungus, M. alternans, which was highly effective in converting DDT to water-soluble rather than hexane-extractable products. These compounds may represent new groups of DDT metabolites which might be generated by pathways of degradation not heretofore characterized. Recently, Miyazaki and Thorsteinson (22) reported that Nitzschia and another diatom converted DDT to DDE and an unidentified water-soluble product.

Preliminary characterization of the M. alternans metabolites revealed that they are not DDA, DBP, DBH, or PCPA (4, 18). On the basis of their mass spectra, these products do not contain chlorine. If indeed devoid of chlorine, the compounds no doubt serve as a carbon or energy source for some microorganism and thus would be decomposed in nature.

Juengst and Alexander (18) investigated the ability of a number of marine microorganisms to convert DDT to water-soluble products. They reported that nearly one-half of the marine isolates examined transformed 5 to 10% of the added DDT to water-soluble products in 8 to 18 days. Nevertheless, when model marine communities were examined, no water-soluble products of DDT metabolism were detected in 5 weeks of incubation. Solubilization was not enhanced when about 80 different carbon sources were added to these model ecosystems in a total of 100 different treatments. It is not yet clear why half of the microorganisms in the waters are able to metabolize DDT, as indicated by pure culture studies, yet the process is not rapid in the natural ecosystem. In this regard, it is of interest that when a DDT-treated field was inoculated with M. alternans, no DDT degradation was apparent (5).

The microbial formation of the benzhydrol ether from benzhydrol raises some interesting and ecologically important questions, particularly since one of the products of microbial metabolism of DDT is DBH, the chlorinated derivative of benzhydrol. If the halogenated analog is biologically converted to the chlorinated benzhydrol ether, it may be more resistant to biodegradation than DDT or DBH or be toxic to a different group of organisms. Furthermore, this finding suggests the possibility that the disappearance of a compound like a benzhydrol may not reflect degradation but rather a dimerization or the formation of some conjugate of the parent compound.

The present report demonstrates that para substituents on one of the two aromatic rings increase the molecule's resistance to biological attack. When both rings had such substituents, the molecule was even more persistent. Although chlorines are known to be associated with resistance, similar results were obtained with hydroxyl, nitro, and amino groups.

Two biochemical mechanisms of ring cleavage are widespread. Ortho-cleaving oxygenases are common but are extremely sensitive to the presence of substituents on the aromatic ring, particularly para-substituted chlorines. Meta-cleaving oxygenases are less common but are not as seriously affected by ring substituents (13). However, meta-cleavage typically yields intensely colored muconic acids (10,13,15), but to date no such muconic acids have been demonstrated to result from DDT metabolism.

Removal of the para-substituted chlorine by a dechlorinase enzyme (16) or by a change in the location of the group to the meta position by the NIH shift (10) may promote DDT biodegradation. It is noteworthy that enzymes often act upon a small group of structurally related substrates. Thus, Cory and Suhadolnik (11) found that adenosine deaminase, which catalyzes the removal of amino groups from adenosine, can also cleave a chlorine located in place of the amino group. The Km for this enzyme was 8×10^{-5} M and 6×10^{-4} M for the amino and chloro compounds, respectively, whereas the Vmax for the chlorinated analog was one-fourth that of the amino substrate.

Since other enzymes removing carbon-linked substituents may exhibit a similar capacity to effect a dechlorination reaction, a number of marine bacteria able to grow on para-substituted aromatic compounds were isolated. Testing of these organisms for dechlorinase activity and their ability to metabolize DDT and its known metabolic products is being conducted.

Attempts to isolate microorganisms able to use para-substituted aromatics as their sole carbon sources were unsuccessful. It may be that these microbes need a series of growth factors in order to grow on aromatics, but Crawford et al. (8) noted that some of the chlorinated and non-chlorinated aromatics they used inhibited microbial growth at levels above 0.025%. Consequently, microorganisms able to use para-substituted aromatics as sole carbon sources may exist, but they may not appear in enrichments containing the usual concentrations of carbon sources.

Enzymes which could catalyze the dechlorination of DDT might not be active in the sea. Thus, if the Km of the putative dechlorinase is high, the para-substituted aromatic would also need to be present in high concentrations for a reasonably rapid reaction to occur, but the chemical may be toxic, insoluble in water, or both. On the other hand, microbial cells may have mechanisms to concentrate DDT to allow a reaction to take place, even though the concentration of DDT in solution is extremely low. Microorganisms able to accomplish this feat must be relatively rare, witness the persistence of the pesticide.

Water-solubility of DDT also probably contributes to its resistance to microbial attack, and its high lipid solubility (2) may further enhance its persistence. Its solubility in water is about 2×10^{-9} M (4). Woodwell et al. (29) estimated the total concentration of DDT in the biota of the world in the late 1960's to 5.4×10^{6} kg. Much of this DDT is associated with lipids, and the accumulation of the pesticide in lipids bound in tissues may protect the insecticide from microbial attack since the tissues are free of microorganisms. When the tissues undergo decomposition, however, the DDT is exposed for microbial attack. Still, if the time needed for decomposition of DDT is long, it

may be reincorporated into the lipids of other organisms before appreciable destruction occurs. Indeed, the failure of natural microbial communities to degrade DDT, while pure cultures were active, may have resulted in a concentration of the chemical by the non-degraders, thereby shielding it from attack.

To help provide a basis for predicting the fate of DDT in marine environments, a number of environmental variables were examined to determine which might influence microbial modification of the insecticide. Hopefully, the results will be of value in anticipating what might take place in estuaries, areas polluted with sewage, warm vs cold seas, and in areas of differing salinity. These factors assume importance since DDT applied to soil probably accumulates in the sea (12,19,29). Once in the ocean, the pesticide residues become associated with organic material (19) and slowly sink into the abyss, where they may persist for thousands of years. If marine microorganisms transform DDT to water-soluble, biodegradable compounds on route to the abyss, such an accumulation would not occur. It is hoped that samples from the abyss can be obtained next year to assess whether DDT or its metabolites are present.

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III. Section 3

A. MATERIALS AND METHODS

Cultures. Inorganic solutions contained diphenylmethane, benzhydrol and analogues of p-chlorophenylacetic acid in order to isolate microorganisms capable of utilizing these substrates as sole sources of carbon and energy. Pseudomonas putida was grown on 0.4% (v/v) diphenylmethane-mineral salts broth containing 10 ml 1 M Na_2HPO_4, 4.0 ml 1 M KH_2PO_4, 10 ml 1 M $(NH_4)_2SO_4$, 1.0 ml 20% $MgSO_4.7H_2O$, 1.0 ml 0.05% $FeSO_4$, 1.0 ml 0.05% $Ca(NO_3)_2$, 4.0 ml diphenylmethane and water to make 1.0 liter (pH 7.0).

Marine model ecosystems. To establish the model ecosystems, surface water was obtained from Sagamore Terrace Beach in Westbrook, Conn., and sediment was collected from three sites in Saybrook and Westbrook, Conn.: gravelly coarse sand from Sagamore Terrace Beach, fine sand from Old Kelsey Point Beach, and fine sandy loam from the mouth of Back River. The surface water and sediments were transported back to the laboratory, and the model ecosystems were established within 48 hr of sample collection. Three model ecosystem types were established, one containing 75 ml of sea water, another 75 ml of sea water plus 50 g sediment, and the last with 75 ml of flooded sediments. These were placed in 125 ml wide-mouth screw-cap bottles, and six separate bottles were used for each variable.

Preparation of samples of model marine ecosystems. Each of these model ecosystems received 3.75 mg of DDT in 0.25 ml acetone. Unless otherwise noted, all bottles were incubated in the dark at 23°C. One of six bottles for each variable was removed at 0, 2, 5, 8, 12 and 27 weeks of incubation for analysis. In some instances, organic carbon was added to the bottles in the form of 25 mg of lyophilized Cylindrospermum cells, 1.0 ml of raw sewage or 0.02 ml of diphenylmethane.

The samples taken for analysis were filtered, and the filtrate was acidified to pH 1.6. Both the filter paper containing sediments and filtrates were extracted with hexane, dried over anhydrous Na_2SO_4 and concentrated to 25 ml. One milliliter of the concentrated hexane extract was used for methylation with 2.0 ml of BCl_3-methanol (Applied Science Laboratories, State College, Pa.). The methylated and nonmethylated products were then extracted with hexane, and the volume was brought to 25 ml. The samples were examined by gas-liquid chromatography without any further clean-up.

Manometry. Standard manometric procedures were used (8). Each Warburg flask received 2 μmoles of substrate in 2.5 ml 0.1 M phosphate buffer in the main compartment, and the side arm received 0.5 ml of cell suspension. In ecosystem respiration experiments, the main vessel received 4.0 g of air-dried Lima loam mixed with 1, 10, 100, or 1000 ppm of DDT analogues and 1.0 ml distilled water, and the center well received 0.2 ml of 20% KOH.

Chlorophyll assay. The algal cells were centrifuged from 5.0 ml of the broth, and the pellet was extracted with 5 ml of 1% diethylether-methanol. The absorbance of the chlorophyll extract was measured at 665 nm using a Beckman DB-G grating spectrophotometer.

Thin-layer chromatography. The products of p-chlorophenylacetic acid cometabolism were separated on chromagram sheet 6060 silica gel containing a fluorescent indicator. The products were separated by two-dimension chromatography using ethanol:water (80:20) and toluene:ethylformate:formic acid (5:4:1) as solvents.

Gas-liquid chromatography. A Varian Aerograph 1740-20 gas chromatograph equipped with ^{63}Ni-electron capture and H_2-flame ionization detectors was used.

Two different Pyrex glass columns of 0.3 X 183 cm length were employed. One of the columns was packed with 3% OV-1 on gas-chrom Q, AW, DMCS, 100/120 mesh, and the other contained 10% DC 200 on gas-chrom Q, AW, DMCS, 100/120 mesh. The operating conditions were 205°C, 180°C, and 260°C for the injector, column, and detector, respectively. The flow of N_2 gas through the column was adjusted to give a retention time for DDT of about 20 min for the OV-1 column and 25 min for the DC 200 column.

Gas-liquid chromatography-mass spectrometry. A Finnigan 3600 series gas chromatograph-mass spectrometer coupled with a PDP-8 data system was used to obtain the mass spectra of various cometabolic products. The gas chromatograph was equipped with a 3% OV-1 glass column.

Infrared spectrometry. Infrared spectra were obtained on 0.5 to 1.0 mg samples incorporated into 50 mg KBr mini-discs using a Beckman IR 10 infrared spectrophotometer.

Silylation of samples for gas chromatography. The cometabolic products of DDT-analogues were silylated by the addition of 0.1 ml Regisil-1 (Regis Chemical Co., Chicago, Ill.) to 1.0 mg of dried sample. The samples were diluted with hexane or diethyl ether.

Chemicals. 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD), 1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene (DDE), bis(p-chlorophenyl)acetic acid (DDA), 4,4'-dichlorobenzophenone (DBP), 4,4'-dichlorobenzhydrol (DBH), diphenylmethane (DPM), and p-chlorophenylacetic acid (PCPA) were obtained from Aldrich Chemical Co., Plainview, N.Y. Bis(p-chlorophenyl)methane (DDM) was purchased from Eastman Organic Chemicals, Rochester, N.Y. 1,1,1',1'-Tetraphenyldimethylether was synthesized by the method of Pratt and Draper (5).

B. RESULTS

Isolation of cometabolic products of DDT-analogues. Pseudomonas putida, an organism capable of utilizing diphenylmethane or benzhydrol as sole source of carbon and energy, was used to detect the formation of products of DDT cometabolism. This bacterium was chosen because of its ability to cometabolize known intermediates of DDT metabolism such as bis(p-chlorophenyl)methane, 4,4'-dichlorobenzhydrol, and 4,4'-dichlorobenzophenone. These compounds and also diphenylmethane, benzhydrol, 1,1,1',1'-tetraphenyldimethylether, bis(p-chlorophenyl)acetic acid, and DDT were provided as substrates in studies of cometabolism. The tetraphenylether, a compound we have found to be produced in diphenylmethane metabolism, was included in order to establish the biological breakdown of this complex ether. The bacteria were grown in large quantities, and resting cells derived from the broth were incubated with the nonchlorinated and chlorinated molecules for 4 and 7 days, respectively. The products were isolated by extraction techniques. The metabolites then were silylated, and mass spectra of each component were obtained. The many products isolated are given in Table 1. There were many products of unknown identities from diphenylmethane, DDM and DBH.

Studies of p-chlorophenylacetic acid cometabolism. In studies of the degradation of p-chlorophenylacetic acid (PCPA), a product of bis(p-chlorophenyl)-methane decomposition, many bacterial isolates were obtained on nonchlorinated analogues of PCPA. These included phenylacetic acid, p-hydroxyphenylacetic acid and p-methoxyphenylacetic acid. Analogues containing amino, nitro or chlorine substituents in the para position were found to be toxic, and no bacterial isolates could be obtained by the methods used. The compounds were

TABLE 1

Products formed in the cometabolism of DDT analogues

by Pseudomonas putida

Substrate	Product
DPM	Benzhydrol, phenylacetic acid
Benzhydrol	Hydroxy benzhydrols
	Phenylglycolic acid
	Hydroxybenzophenone (?)
1,1,1',1'-Tetraphenyldimethyl ether	Benzhydrol
	Benzophenone
DDM	Benzophenone
	DBH
	DBP
DBH	Benzophenone
	Benzhydrol
	DBP
DDA	DDM
	DBH
	DBP
DDT	One unidentified product

toxic even at a concentration of 100 µg/ml. Resting cells of one of the bacterial isolates obtained on phenylacetic acid consumed 1.56 µmoles of O_2 per µmole of PCPA in Warburg respirometers, indicating its ability to cometabolize PCPA. This bacterium was grown in a medium containing 0.1% phenylacetic acid, and resting cells free of residual phenylacetic acid were obtained by washing with 0.1 M phosphate buffer (pH 7.0). These cells were incubated with PCPA for 6 hours, after which the culture supernatant was extracted with diethyl-ether. The resultant concentrated ether extract was placed on thin-layer plates and developed in toluene:ethyl formate:formic acid (5:4:1). Two products of PCPA metabolism were detected, and the spots corresponding to these products were scraped from the TLC plates and dissolved separately in ether. A portion of these components were silylated and subjected to GLC-mass spectrometry. One of the products was found to be a monohydroxylated chlorophenylacetic acid. Work is underway to determine whether the hydroxyl group is located on the ring or the side chain of the molecule.

Effect of environmental conditions on DDT degradation in model marine ecosystems. To provide a basis for predicting the fate of DDT in the ocean, a number of environmental variables were examined to determine which might influence microbial modification of the insecticide. The variables studied include salinity, temperature, oxygen tension, presence of sediment, and availability of various organic nutrients and algal cells. Analysis for the products accumulating in the various test ecosystems revealed no metabolites in the surface water: (a) incubated at 4°, 15°, 23° or 32°; (b) having salinities equivalent to specific gravities of 1.015, 1.022 or 1.035; (c) maintained under anaerobiosis or under aerobic conditions; or (d) amended with sewage or

with diphenylmethane at regular intervals. It is evident from the summary presented in Table 2 that products did accumulate in some of the model ecosystems, however. In model ecosystems receiving the alga Cylindrospermum sp. or diphenylmethane (a structural analogue of DDT), DDD, DDE and DBP were formed from DDT.

DDD was found in every instance where Cylindrospermum sp. was added to model ecosystems. In the surface waters containing the alga and in surface water-fine sandy loam model ecosystems, about 2 to 11% of the DDT was converted to DDD. By contrast, no conversion of DDT to DDD was evident in the unamended surface water-sediment model ecosystems. Not only the extent but also the rate of DDD formation in the presence of algae was affected by the type of sediment.

Supplementation with diphenylmethane as one or as a series of amendments resulted in nearly identical rates of conversion of DDT to DDD. Addition of sewage to the surface water-fine sandy loam ecosystem also stimulated the production of DDD. The conversion of DDT to DDE took place in the surface water-fine sandy loam ecosystem. After 12 weeks, DDE was detected in the samples amended with Cylindrospermum sp. cells or sewage, but only the latter markedly stimulated this conversion.

In the flooded sediment, the average rates of DDD formation during the first 5 weeks of incubation were 142, 92, and 52 µg/week for the flooded fine sand, fine sandy loam, and the gravelly coarse sand, respectively. In the gravelly coarse sand, the rates of disappearance of DDT and of appearance of DDD were nearly identical. In addition to DDD and DDE, a compound with the chromatographic characteristics of DBP was detected in waters receiving the insecticide (Table 3). However, a chromatographically similar compound was present in the sample at 0 time so that the compound may merely be a constit-

TABLE 2

Products of DDT in model marine ecosystems

Test ecosystem	Amendment	Product detected
Surface water	*Cylindrospermum* cells	DDD
Surface water and	None	None
fine sand	Sewage	None
	Cylindrospermum cells	DDD
	DPM[a]	None
	DPM[b]	DDD, DBP
Surface water and	None	DDD
fine sandy loam	Sewage	DDD, DDE, DBP
	Cylindrospermum cells	DDD, DDE
	DPM[a]	DDD, DBP
	DPM[b]	DDD
Flooded sediments:		
Gravelly coarse sand	None	DDD, DBP
Fine sand	None	DDD, DBP
Fine sandy loam	None	DDD, DBP

[a]Added at the beginning of incubation.

[b]Added at 0, 2, 5, 8, and 12 weeks.

uent of the water and/or the sediment (Table 3). On the other hand, the level of this compound increased markedly in two of the incubated flooded sediments, suggesting that the unknown may indeed be DBP.

Ecological effects of DDT and its analogues. Studies on the ecological effects of DDT and its products were carried out by measuring respiratory activities of samples of natural microbial communities and by assessing the effect on algal productivity (expressed as the change in chlorophyll content of the total biomass) using Chlorella vulgaris as the test organism. DDD, DBP, PCPA, and DDT had no effect on community respiration at concentrations up to 100 ppm. DDE, DDM, DBH, and DDA inhibited oxygen uptake considerably at concentrations of 100 ppm and above. There was a stimulatory effect on community respiration rate with DDM and DBH at concentrations up to 10 ppm.

Neither the intermediates of DDT degradation nor DDT itself had an adverse effect on algal productivity. Conversely, bis(p-chlorophenyl)methane, 4,4'-dichlorobenzophenone, 4,4'-dichlorobenzhydrol, bis(p-chlorophenyl)acetic acid, PCPA and DDT enhanced algal productivity.

C. DISCUSSION

Wedemeyer (9,10) was the first to report the sequential degradation of DDA to DDM, DBH and DBP using whole cells or cell-free extracts of Klebsiella. The ring cleavage of bis(p-chlorophenyl) methane to p-chlorophenylacetic acid was the first case of extensive degradation of DDT, and this was reported by Focht and Alexander (2). The present data on chlorinated intermediates confirm the pathway:

$$\text{DDA} \rightarrow \text{DDM} \rightarrow \text{DBH} \rightarrow \text{DBP}.$$

The formation of benzyhdrol and benzophenone from chlorinated products generated from DDT indicates a direct dechlorination of 4,4'-dichlorobenzhydrol

TABLE 3

Apparent DBP formation in model marine ecosystems

Model ecosystem	Weeks					
	0	2	5	8	12	27
	Apparent DBP formed, µg					
Surface water (control)	0.4	0.7	0.4	0.4	0.3	0.4
Surface water + algae	43	11	34	26	0.8	2
Surface water + fine sand						
+ sewage	22	8	10	9	0.7	2
+ algae	6	40	68	5	5	5
+ DPM	38	22	20	0.3	9	13
Surface water + fine sandy loam	31	7	5	5	7	24
+ sewage	100	6	14	49	215	7
+ algae	28	110	42	3	7	21
+ DPM	55	18	9	28	57	7
Flooded sediments						
Gravelly coarse sand	30	66	98	85	80	45
Fine sand	469	1167	1845	2369	2534	154
Fine sandy loam	1139	1557	2492	1347	1241	726

and 4,4'-dichlorobenzophenone. This is the first report of such dehalogenation of ring chlorines derived from DDT. Identification of the many products from nonchlorinated analogues (Table 1) suggests the pathways shown in Figure 1. It appears that Pseudomonas putida has an array of enzymes or a multifunctional oxidase system capable of carrying out ring hydroxylations, side-chain hydroxylations, and ring cleavages. An analogy exists between the oxidation of diphenyl methane to benzophenone with the hypothetical reaction sequence involving phenylacetic acid, phenylglycolic acid and phenylglyoxylic acid. Focht and Alexander (2) found phenylacetic acid (PA) and phenylglyoxylic acid in the culture supernatants of Hydrogenomonas sp. A nonspecific oxygenase may be involved in the conversion of DDM to DBH to DBP; DPM to BH to BP; and PA to PGA to PGO. The hydroxylation of p-chlorophenylacetic acid has not been reported previously, and it seems possible that microorganisms are able to cleave the ring and detoxify the molecule.

Patil et al. (4) reported that no products were found when DDT was added to surface water from the open sea, estuaries, or shores of the Hawaiian Islands. The present results with surface water from Long Island Sound exposed to a number of different environmental conditions confirm their findings, except that DDT was slowly converted to DDD in surface water to which algal cells were added. Woodwell et al. (11) proposed that DDT transported to the sea associates with algae and eventually is carried to the abyss as the dead algae settle. The present findings on DDT metabolism in the presence of dead Cylindrospermum sp. indicate that microorganisms associated with the nonliving algae may convert a substantial quantity of DDT to DDD, the latter then accumulating. Consequently, DDD might be expected to build up in the abyss along with DDT. The production of DBP in flooded sediments and in other model ecosystems suggests that some of the DDD

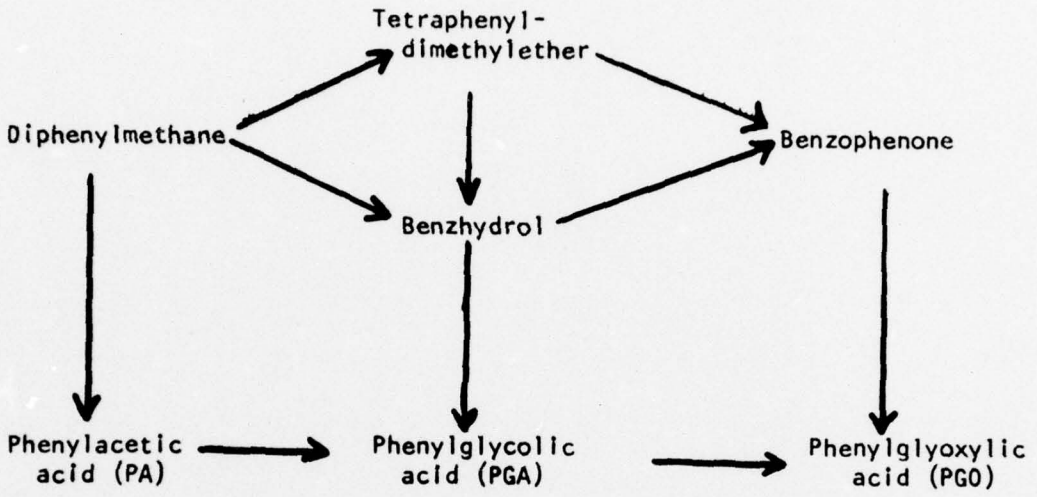

Fig. 1. Pathway for the metabolism and degradation of DDT analogues.

can be further metabolized, presumably via the previously described pathway to yield DBP (3). The data show that the type of sediment and organic material govern the rate of DDD generation. Thus, phytoplankton or carbonaceous materials undergoing decay and possibly anaerobiosis may promote the transformation in the sea.

Supplementation with diphenylmethane as one or as a series of amendments resulted in conversion of DDT to DDD. Since diphenylmethane resembles DDT structurally, it may induce enzymes for DDT metabolism, induce transport mechanisms for its entry into cells or, because diphenylmethane can serve as a source of carbon and energy to some microorganisms, it may stimulate microorganisms particularly active in the transformation. In the flooded fine sandy loam, more DDT was recovered at latter periods of incubation than at earlier intervals. It is known that DDT can bind to organic compounds (7), bacterial cells, and algae (6), the bound DDT possibly becoming less extractable than the free chemical, and the greater recovery may reflect destruction of the organic material or cells retaining the chemical in a less extractable form.

Studies on effects of DDT and its various metabolic products revealed no significant influence on microbial community respiration and algal productivity. Although it is possible that the products of degradation are more toxic than the parent molecule to one or more members of an ecosystem (1), the present investigations suggested that none of the products is toxic to microorganisms at concentrations even higher than those usually found in the marine environment.

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IV. Section 4

A. MATERIALS AND METHODS

Bacteria. Pseudomonas putida 1, Enterobacter 2 and Pseudomonas 3 were obtained from enrichments containing diphenylmethane as sole source of carbon. Pseudomonas strains 4 and 7 and Alcaligenes 5 were able to utilize benzhydrol as sole source of carbon. The mineral salts solution used in preparing the enrichments and for testing the degradability of DDT metabolites is described by Pfaender and Alexander (16). The bacteria were identified by comparing their morphological, cultural and biochemical characteristics with described bacteria (5).

Fungi. Eleven fungi were obtained from the culture collection of the Laboratory of Soil Microbiology, Cornell University. Mucor alternans, an organism capable of converting DDT to water-soluble metabolites (1), was kindly provided by Dr. E. P. Lichtenstein, University of Wisconsin, Madison. All the fungi were maintained on potato dextrose agar (Difco) slants and were tested for their ability to degrade DDT or its metabolites.

Preparation of trimethylsilyl (TMS) ether derivatives. Ether solutions containing 1 to 2 mg of products were placed in small vials covered with screw-caps fitted with rubber septa reinforced with nylon and backed with Teflon film. The ether was evaporated under a stream of dry N_2. The samples were dissolved in a few drops of hexanes or pyridine dried over 13 X molecular sieves (Fisher). Regisil-2 (Regis Chemical Co., Chicago, Ill.) was added to the samples at a rate of 0.1 ml/mg of chemical, and the tightly capped vials were left overnight in the dark at 20 C or for 4 h at 60 C. The reaction mixture was evaporated under a stream of N_2, redissolved in ether or hexanes and injected onto the column of a gas-liquid chromatograph.

Gas-liquid chromatography. A Varian Aerograph gas-liquid chromatograph, model 1740-20 (Varian Associates, Palo Alto, Cal.), equipped with a flame ionization detector and containing a 183 cm x 0.3 cm coiled Pyrex glass column packed with 3% OV-1 coated on acid washed dimethylchlorosilane-treated, 100/120 mesh Gas-Chrom Q (Applied Science Laboratories) was used. The temperature of the column was programmed from 110 to 180 C at a rate of 20 C/min. The temperatures of the injector and detector were 225 C and 240 C, respectively. The gas flow rates were 80, 50 and 400 ml/min for carrier gas (N_2), H_2, and air, respectively.

Mass spectrometry. Mass spectra of products in solutions were obtained with a Finnigan-3300 gas chromatograph-mass spectrometer (GC-MS) equipped with a Systems-150 data processor. The sample spectra were scanned at a rate of one scan per second. The ionization voltage in the mass spectrometer was 70 eV. The gas chromatograph of the coupled GC-MS system was equipped with a 0.3 cm X 305 cm long U-shaped glass column filled with 3% OV-1 coated on 100/20 mesh Gas-Chrom Q. The injector temperature was 250 C, and the column was programmed either from 150 to 200 C at a rate of 10 C/min or from 110 to 180 C at 20 C/min.

Chemicals. Analogues of DDT and biphenyl of highest purity were obtained from Aldrich Chemical Co., Milwaukee, Wisc.

Respirometry. To obtain the resting cells, Enterobacter 2 or Pseudomonas strains 3 or 4 were grown in one liter of 0.5% glucose-mineral salts broth. Cultures of P. putida 1 and Pseudomonas 7 were grown in one liter of trypticase soy broth (BBL) prepared at half of its recommended concentration. Alcaligenes 5 cells were grown in one liter of 0.1% BH-mineral salts broth. The cultures were grown in 2-liter Erlenmeyer flasks incubated on a NBS gyro-

tory shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) operating at 180 rpm for 48 h at 30 C. The cells were harvested by centrifugation, washed six times in 0.1 M phosphate buffer, and resuspended in buffer. The weight of resting cells added per respirometer flask were: 17.0 mg for P. putida 1, 9.0 mg for Pseudomonas 3, 12.6 mg for Pseudomonas 4, 7.7 mg for Pseudomonas 7, 10.8 mg for Enterobacter 2, and 14.0 mg for Alcaligenes 5.

Cometabolism of DDT and its metabolites by bacteria. The reaction medium consisted of 50 ml of 0.1% substrate-mineral salts broth contained in 125 ml Erlenmeyer flasks. The flasks containing DDT also received 500 ng of uniformly ring-labelled ^{14}C-DDT (New England Nuclear, Boston, Mass.) of specific activity 17.86 μCi/mg. Control flasks containing 50 ml of mineral salts broth inoculated with the bacterial cells were maintained. Three 125-ml flasks each containing 50 ml of uninoculated 0.1% substrate-mineral salts broth were included as controls for nonbiological degradation of each DDT metabolite. The growth media for obtaining resting cells of bacterial isolates and the amount of resting cells added into the reaction solutions are presented in Table 1. Cometabolism of DDT was tested with only four bacterial isolates, P. putida, Pseudomonas strains 3 and 7, and Alcaligenes 6.

The bacteria were incubated in media containing the substrates for seven days, and the solutions were acidified to pH 2.0. The reaction mixtures were extracted three times in separatory funnels, once with a solvent mixture containing 15 ml of hexanes and 10 ml of diethyl ether and twice with 20 ml of diethyl ether. The three extracts were pooled, dried and concentrated to 10 ml. One milliliter of this concentrated extract was used for silylation with Regisil-2, and the samples were analyzed by gas-liquid chromatography. A 3% OV-1 column maintained initially at an oven temperature of 110 C and

TABLE 1. Bacteria used in the cometabolic studies on DDT metabolites.

Organism	Dry weight of resting cells added per 50 ml cometabolic medium, mg
Pseudomonas putida 1[a]	98
Pseudomonas 3[a]	115
Pseudomonas 4[b]	52
Alcaligenes 5[b]	42
Alcaligenes 6[b]	47
Pseudomonas 7[b]	56

[a] The resting cells were obtained by growing the cultures in 0.05% DPM + 0.1% glucose-mineral salts broth.

[b] The resting cells were obtained by growing the cultures in 0.05% BH + 0.1% glucose-mineral salts broth.

then programmed to 180 C at a rate of 20 C/min was used for analysis of the products. The samples indicating new peaks on gas chromatographic recorder traces were analyzed by GC-MS.

The aqueous portions remaining after extraction of the DDT-salts broth were saved. One-milliliter portions of these were added to scintillation vials containing 10 ml of scintillation cocktail. The cocktail was prepared by mixing 5 g of PPO (2,5-diphenyloxazole, Fisher), 100 g of naphthalene and dioxane to make one liter. The radioactivity of the samples was counted on a Beckman LS-100 liquid scintillation system.

Cometabolism of DDT and its metabolites by fungi. Twelve different fungi were used: Aspergillus flavus strains 141 and 147, A. niger, A. conicus, Penicillium brefeldianum, Schizophyllum sp., Pycnidiophora dispersa, Cercospora oryzae, Pyricularia oryzae, Thanatephorus cucumeris, Mucor alternans and a false smut fungus of rice. The cometabolic substrates, the concentrations used in the mineral salts broth and the amount of medium used per flask were the same as in studies with the bacteria. Each fungus was grown for 5 days in 500 ml of 0.5% glucose-mineral salts broth of pH 6.0 amended with 4.0 g of nutrient broth powder (Difco). The fungi were washed free of nutrients, and the mycelial mats were homogenized by using a Servall Omni-mixer. The fungal mycelia were taken up in 50 ml of 0.1 M phosphate buffer of pH 7.2 and used as an inoculum by adding 8 ml of suspended mycelia (48 ± 5 mg on a dry weight basis) for each flask containing 50 ml of 0.1% DDT metabolite-mineral salts broth. The fungi were incubated for 7 days at 30 C on a rotary shaker. The contents of the flasks were extracted after adjusting the pH to 2.0. The extraction procedure was similar to the one used in the bacterial studies, except that the mycelial mats were on Whatman No. 1 filter

paper and extracted separately. The mycelial mats collected on the filter paper were extracted by shaking with a solvent mixture containing 15 ml of hexanes and-10 ml of diethyl ether in a 50-ml Erlenmeyer flask covered with aluminum foil. The flasks were shaken on a rotary shaker for 2 h. The solvent portion was separated by filtering through Whatman No. 1 filter paper and processed along with the solvent portions obtained during the extraction of reaction supernatants. The solvent portions were pooled, dried over anhydrous Na_2SO_4, and concentrated by flash evaporation to 10 ml. A one-milliliter portion of each extract was used for silylation. The silylated portion was diluted to 10 ml with ether, and 1.0 µl of this was used for injection onto a 3% OV-1 column of a gas chromatograph. The samples showing evidence for new products were analyzed by GC-MS.

Products from p-chlorophenylacetic acid. A strain of Arthrobacter able to use phenylacetate was isolated from lake water using an inorganic salts solution supplemented with 0.10% of the carbon source. When resting cell suspensions of the bacterium from a 48-h culture were incubated for 4 h with 2.0 µmol of p-chlorophenylacetate in the Warburg apparatus, 4.8 µmol of O_2 were consumed.

A 300-ml portion of cell suspension was incubated with 200 µmol of p-chlorophenylacetate on a rotary shaker at 28 C for 4 h, the cells were removed by centrifugation, the supernatant fluid was acidified to pH 2.0 with HCl, and the solution was then extracted with ether after adding 10 to 15% NaCl. Portions of the ether extract were dried under a stream of N_2, and then 0.2 ml of bis(trimethylsilyl)trifluoro acetamide was added. After 30 min, about 0.5 to 1.0 ml of ether was added, and the sample was injected into a Varian 1700 gas chromatograph equipped with a flame ionization de-

tector and a 1.8 m X 0.32 cm column containing 3% OV-1 on Gas Chrom-Q, 100/120 mesh (Applied Science Laboratories, State College, Pa.). The operating temperatures were 250, 150, and 260 C for the injector, column, and detector, respectively. The carrier gas was N_2 at a flow rate of 30-35 ml/min. Later, the products were subjected to GC-MS.

B. RESULTS

Evaluation of microbial ability to degrade DDT intermediates and their analogues by Warburg respirometry. During the preliminary oxygen uptake experiments, the resting cells were obtained by growing them in 0.5% diphenylmethane (DPM)-mineral salts broth or 0.1% benzhydrol (BH)-mineral salts broth. The organisms thus obtained showed high endogenous respiration, which might have masked any activity on chlorinated molecules. One likely substrate for the high endogenous oxygen consumption was the oily DPM or insoluble crystals of BH which could not be removed easily from the cells during washings with phosphate buffer. The bacteria were therefore grown in either 0.5% glucose salts broth or half-strength trypticase soy broth. Resting cells of Alcaligenes 5 obtained from 0.1% BH-salts broth exhibited low endogenous respiration (4 µmoles of 0_2 in 5 h).

The oxygen consumption values reported below are averages of two replicated treatments and are corrected for endogenous respiration.

The oxidation of DDT metabolites and their analogues by P. putida is presented in Fig. 1 and Fig. 2. The resting cells obtained on trypticase soy broth were able to metabolize DPM, BH, and benzophenone (BP) (Fig. 1) without an apparent lag period, indicating that the enzyme systems involved are constitutive. The oxygen uptake in the presence of p,p'-dichlorodiphenylmethane (DDM) and p,p'-dichlorobenzhydrol (DBH) was approximately one µmole,

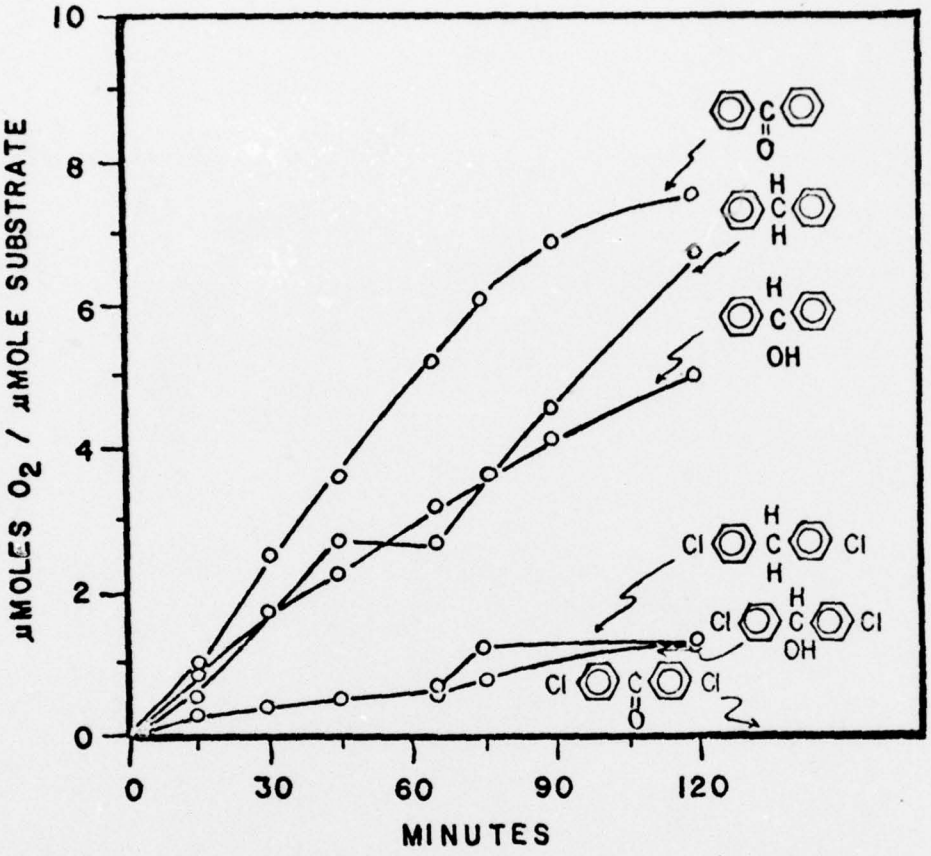

FIG. 1. Metabolism of DDT metabolites and their analogues by P. putida.

indicating the possibility either of cometabolism or a stimulation of endogenous respiration. If cometabolism was involved, it should be possible to isolate the suspected hydroxylated or ring cleavage products from the solutions in the Warburg flasks. No cometabolism of p,p'-dichlorobenzophenone (DBP) was observed.

p-Chlorophenylacetate (PCPA) and p-chlorobenzoate (PCBA) have been reported as products of DDT metabolism (8,9,10,16). These compounds and their nonchlorinated analogues were tested for oxidation by resting cells of P. putida in respirometers. The uptake of oxygen was very high with phenylacetate (PA) but not with benzoic acid (Fig. 2). The biphasic activity with PA is noteworthy. Cometabolism or stimulation of endogenous respiratory activity was observed with PCBA. Oxygen uptake was not noted with PCPA as substrate.

Figure 3 depicts the oxygen uptake by Enterobacter sp. on various analogues of DDM. The oxidative enzymes involved were found to be constitutive. Glucose was completely oxidized (6 µmoles of O_2/µmole of glucose) by Enterobacter 2 in 7 h. A biphasic activity was observed during the oxidation of DPM. Cometabolism was suspected with p-hydroxydiphenylmethane, bis(p-hydroxyphenyl)methane and DDM because of an oxygen consumption of approximately 1 to 2 µmoles with these substrates. An apparent but slight stimulation of endogenous respiration was observed with bis(p-nitrophenyl)methane.

The metabolism of DDM analogues by resting cells of Pseudomonas 3 is presented in Fig. 4. Though the organism was isolated with DPM as sole carbon source, the resting cells consumed only 1.5 µmoles of O_2/µmole of this substrate. Extensive oxygen uptake was observed with p-hydroxydiphenylmethane and bis(p-hydroxydiphenyl)methane. The organism was found to cometabolize

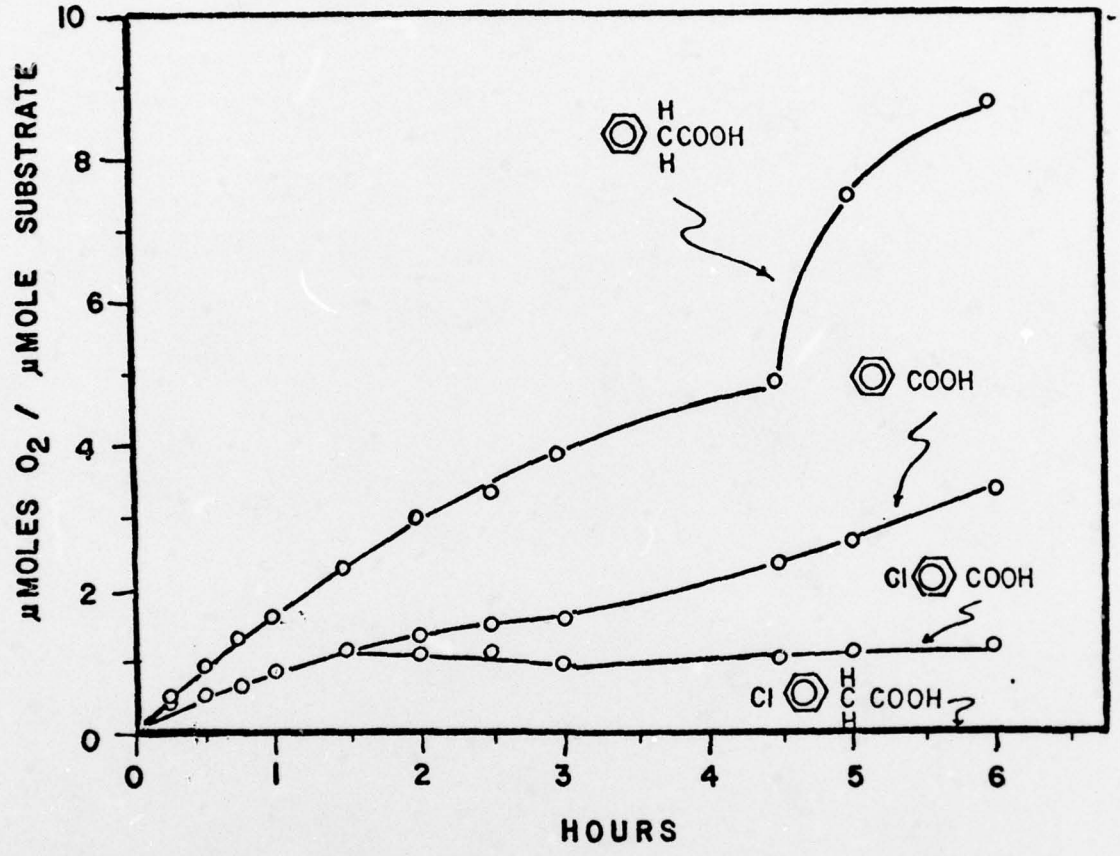

FIG. 2. Metabolism of PCPA and PCBA and their non-chlorinated analogues by P. putida.

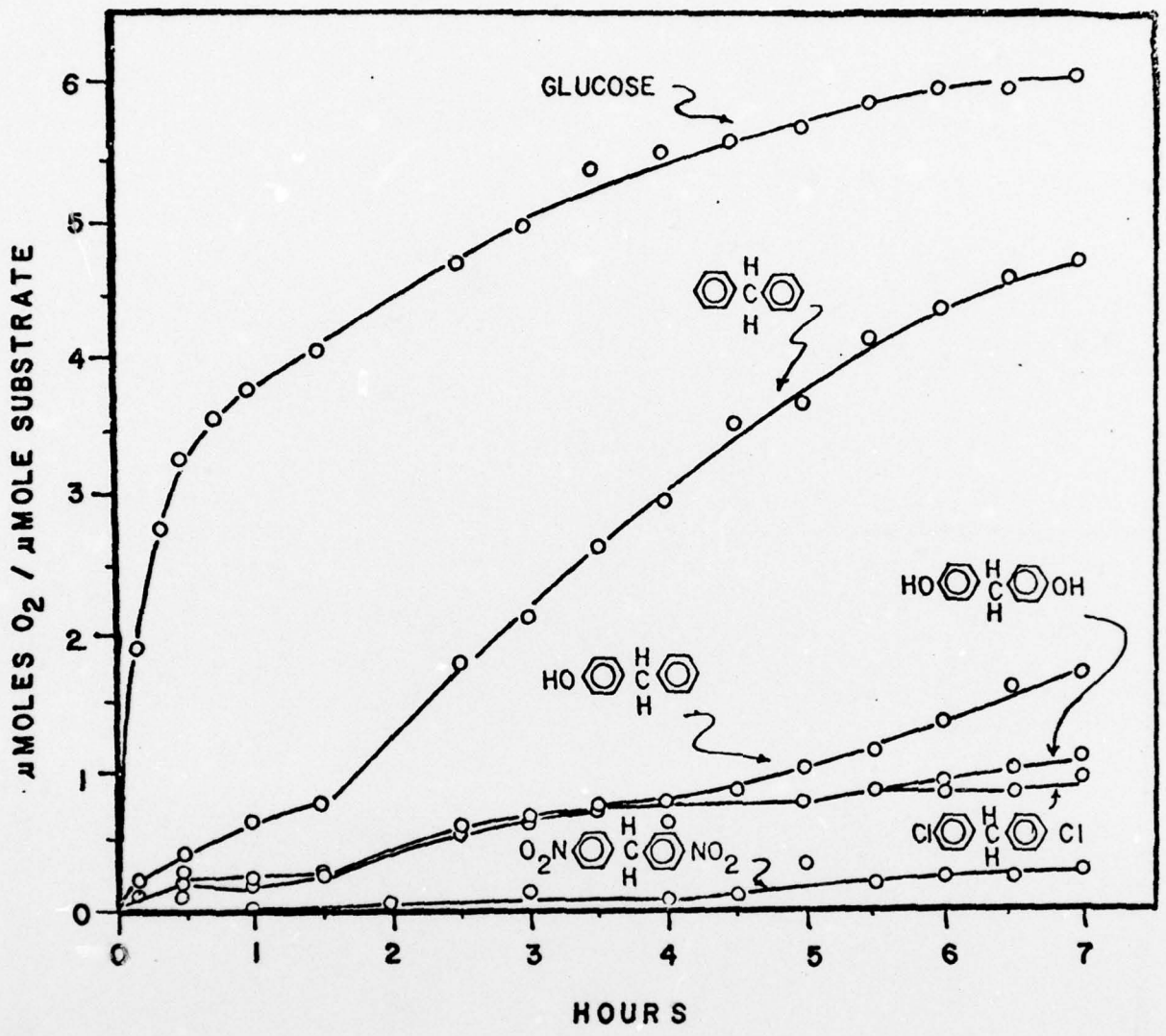

FIG. 3. Metabolism of DDM analogues and glucose by resting cells of Enterobacter 2 grown on 0.5% glucose-mineral salts broth.

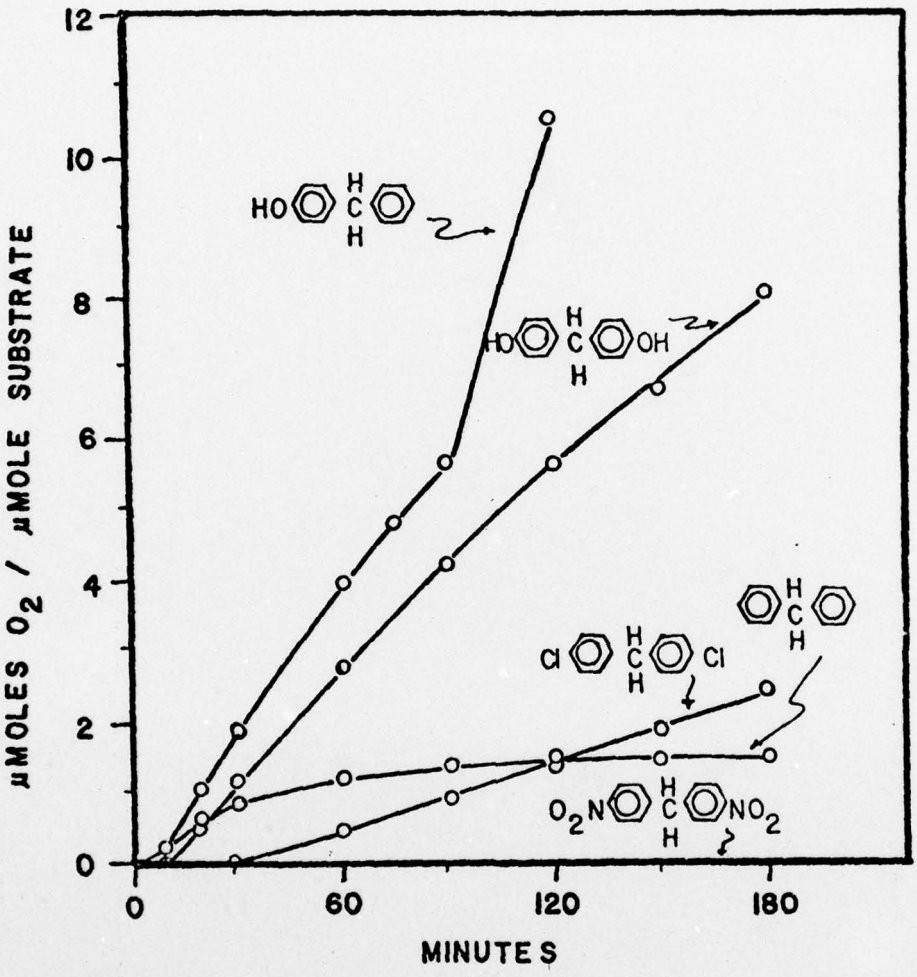

FIG. 4. Metabolism of DDM analogues by resting cells of Pseudomonas 3.

DDM after a lag period of 30 min. Bis(p-nitrophenyl)methane did not stimulate the endogenous respiratory activity.

Resting cells of Pseudomonas 4, which was isolated with BH as sole carbon source, extensively oxidized glucose, BH + glucose, and BH (Fig. 5). The oxidation of p-chlorobenzhydrol, DBH and DPM follow cometabolic patterns. The flask containing one micromole each of glucose and BH had shown a rate of oxidation (14.8 μmoles of 0_2/μmole of substrate/h) very close to the combined uptake rate of BH (4.1 μmoles of 0_2/μmole of substrate/h) and glucose (11.8 μmoles of 0_2/μmole of substrate/h). A biphasic oxidative pattern was observed with BH.

Resting cells of Alcaligenes 5, which was obtained with BH as sole source of carbon, oxidized BH and BP without any lag period (Fig. 6). Cometabolism was suspected with DDM, p,p'-dichlorodiphenylacetic acid (DDA), DBH, DBP and DPM. In other manometric studies (Fig. 7) using the same organism, there was an indication of cometabolism of PCPA and PCBA in that the resting cells of Alcaligenes 5 consumed 1.5 μmoles of 0_2/μmole of these substrates. DDT and methoxychlor were not oxidized, and the observed oxidative activity with these compounds was similar to the endogenous respiratory rate.

The oxidation of various DDT metabolites and their nonchlorinated analogues by Pseudomonas 7 is shown in Fig. 8. The oxidative activity of the resting cells was highest with BH, BP and DBH. The enzymes involved were constitutive. A lag period of 45 min was observed with DPM, indicating the need for enzyme induction. This is the sole instance of enzyme induction in all the studies with different bacterial isolates. Cometabolism was observed with several of the chlorinated molecules.

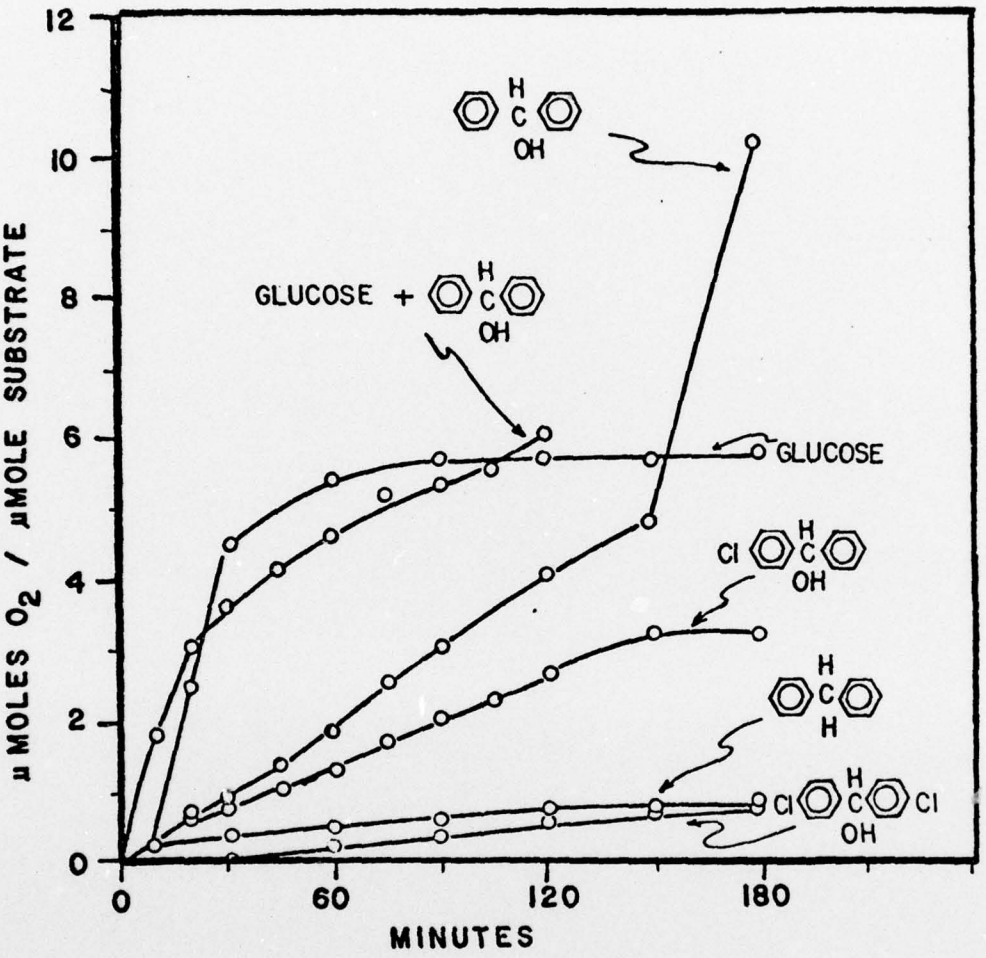

FIG. 5. Metabolism of DBH analogues by resting cells of Pseudomonas 4.

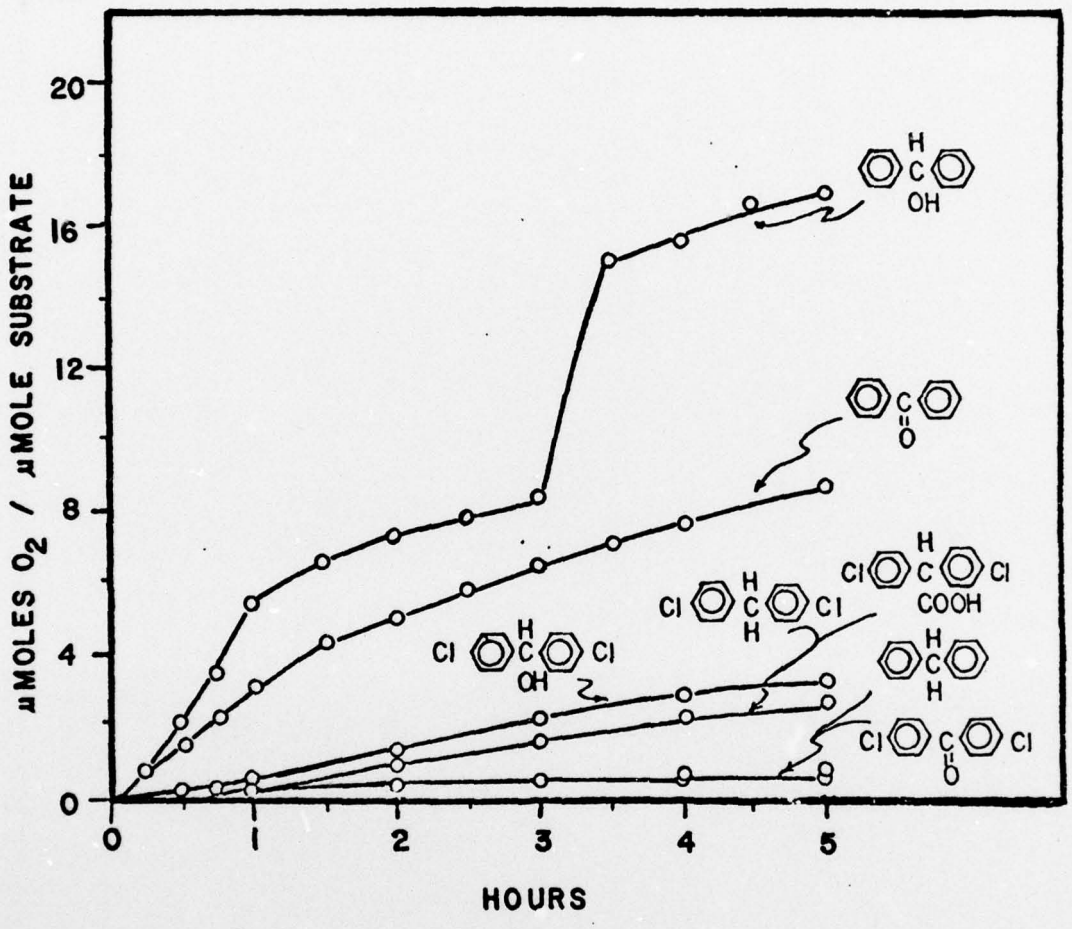

FIG. 6. Metabolism of DDT metabolites and their non-chlorinated analogues by resting cells of Alcaligenes 5.

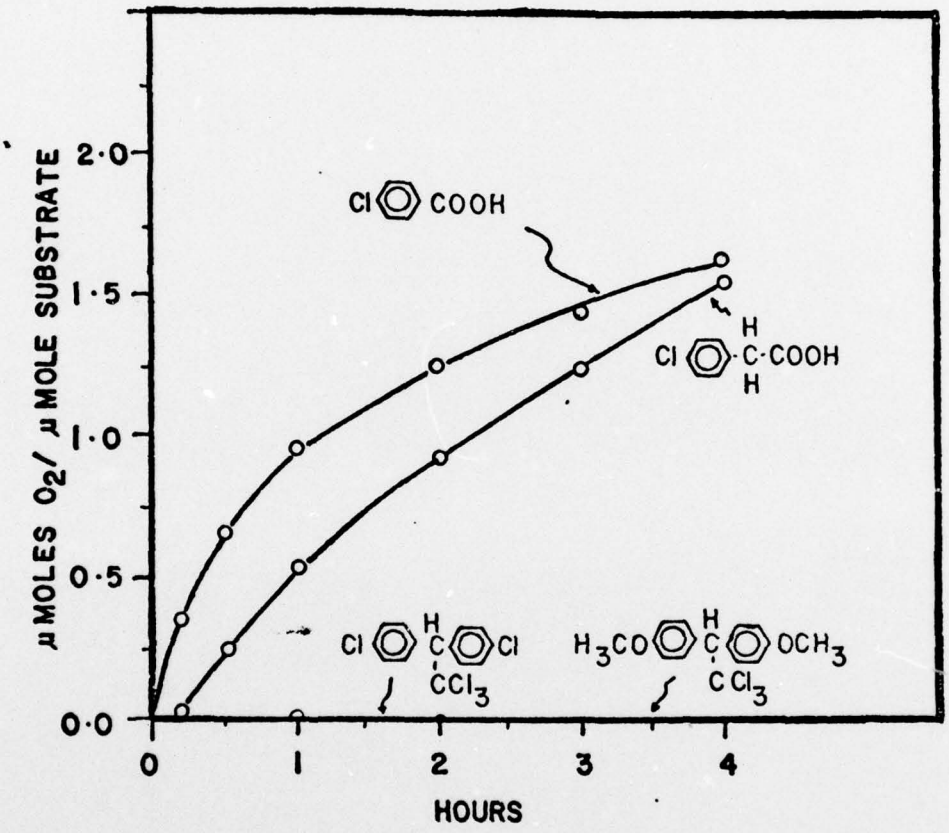

FIG. 7. Metabolism of DDT, methoxychlor, PCPA and PCBA by the resting cells of Alcaligenes 5.

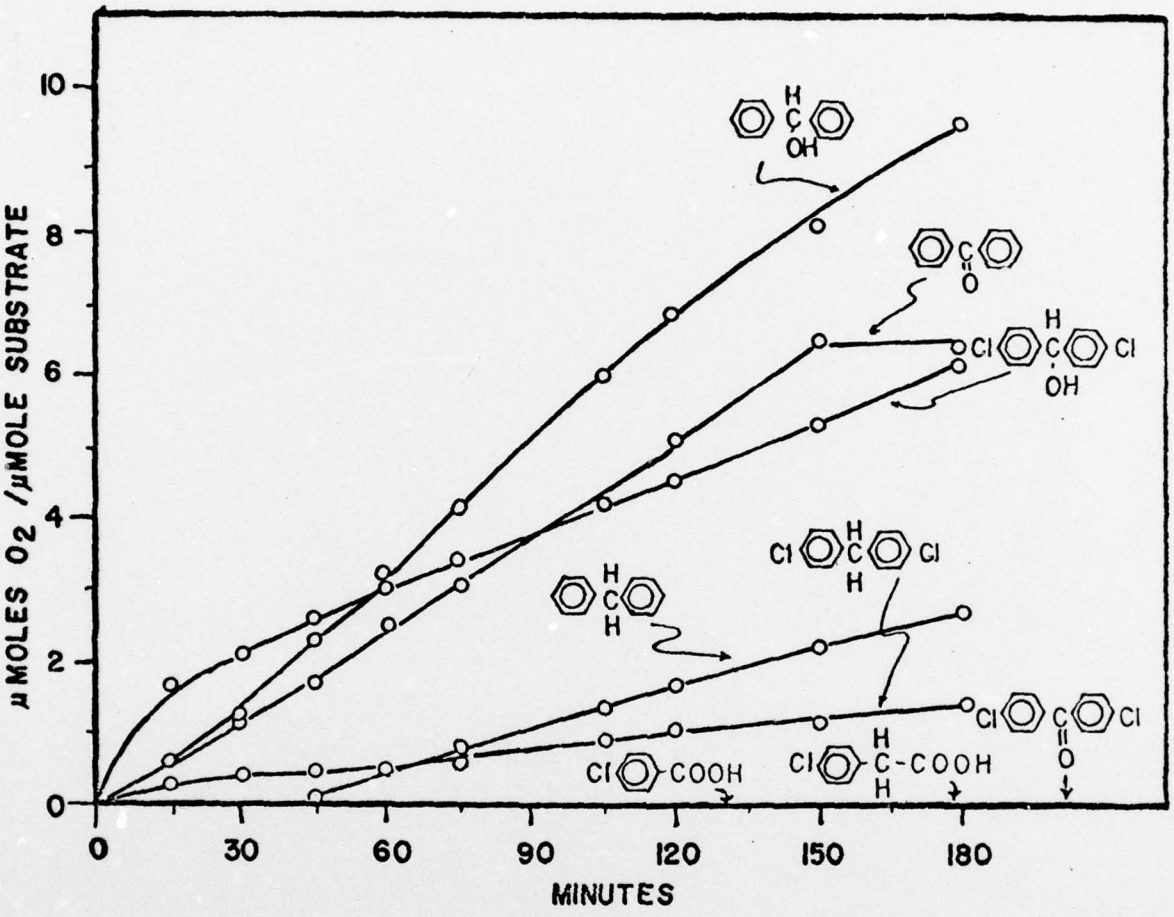

FIG. 8. Metabolism of DDT metabolites and their analogues by resting cells of Pseudomonas 7 grown on trypticase soy broth.

Bacterial cometabolism of DDT and its metabolites. The component products were extracted and analyzed by gas chromatography (GC), and their concentrations were expressed as a percentage of the substrate added. The values reported were averages of duplicate GC analyses and were corrected for extraction efficiencies. Some of the samples were checked at random by GC-MS to confirm the identity of the various compounds detected. The efficiency of extraction of various DDT metabolites is given in Table 2.

During the cometabolism of DDT by 4 bacteria, 1.6 to 16.4% of the added DDT was converted to various products (Table 3). The products identified during the cometabolism of DDT were DDD, DDE, DDM, DBH, and DBP. None of the products reported was detected in controls containing bacterial cells or in uninoculated controls with only DDT. The amount of products detected in the aqueous phase indicates either that DDT had been cleaved to water-soluble metabolites which could not be extracted by the extraction techniques employed or that DDT might have been retained in the slimy material produced by the bacteria. DDD and DDE were the major products detected, and DBP was a less abundant metabolite.

The cometabolic products obtained from the metabolites of DDT are presented in Tables 4 to 7. The compounds detected during the cometabolism of DDA by strains of Pseudomonas and Alcaligenes were DDM, DBH, and DBP (Table 4). Large amounts of DDA could not be accounted for during its degradation by bacteria. This was probably a result of the adsorption of DDA or its products by the bacterial slime. It might also be possible that DDA was totally converted to water-soluble metabolites, which could not be detected. With DDM as a substrate, DBH and DBP were produced, and a considerable amount of products could not be extracted from the aqueous phase (Table 5). DBP was the only product

TABLE 2. Efficiency of extraction for DDT metabolites.

Metabolite	Extraction efficiency[a], per cent[b]
DDM	88 ± 2
DBP	91 ± 3
DBH-TMS[c]	92 ± 1
DDA-TMS	87 ± 4
DDT	99 ± 1

[a] Efficiency of extraction was determined by adding a known amount of the chemical to mineral salts solutions and then re-extracting the compound according to the procedures mentioned earlier in the section on methods. The samples were subjected to GC analysis and the total amount of compound recovered was calculated from the standard curves prepared for each chemical.

[b] Values are averages of three replicate samples

[c] DBH and DDA were analyzed as the trimethylsilyl ether derivatives.

TABLE 3. Cometabolism of DDT by bacteria.

Organism	Per cent products detected[a]							DDT recovered, per cent
	DDM	DBH	DBP	DDE	DDD	Water soluble fraction[b]	Other unidenti-products	
Pseudomonas putida 1	0.0	0.0	0.0	0.0	0.0	1.5	0.1	98.4
Pseudomonas 3	0.4	0.2	0.0	0.6	1.6	2.7	9.1	85.4
Alcaligenes 6	0.0	0.0	0.8	1.0	2.8	2.0	3.8	89.6
Pseudomonas 7	0.0	0.0	0.2	0.6	1.0	1.1	13.5	83.6

[a] Expressed as per cent of total DDT added.

[b] Calculations were based on the amount of radioactivity detected in aqueous phase left over after extractions.

TABLE 4. Cometabolism of DDA by bacteria.

Organism	Per cent products detected[a]				DDA recovered, per cent
	DDM	DBH	DBP	Other[b]	
Pseudomonas putida 1	0.6	0.0	0.0	28.0	71.4
Pseudomonas 3	0.4	0.2	0.0	17.4	82.0
Pseudomonas 4	0.4	2.0	0.0	8.2	89.4
Alcaligenes 5	1.2	1.2	2.6	21.2	73.8
Alcaligenes 6	0.0	0.0	0.0	10.0	90.0
Pseudomonas 7	3.6	0.8	0.0	24.8	70.8

[a] Expressed as per cent of total DDA added.

[b] Indicates the amount of products not identified.

TABLE 5. Cometabolism of DDM by bacteria.

Organism	Per cent products detected[a]			DDM recovered, per cent
	DBH	DBP	Other[b]	
Pseudomonas putida 1	3.6	0.1	5.9	90.4
Pseudomonas 3	0.0	2.4	2.6	95.0
Pseudomonas 4	0.0	0.2	4.8	95.0
Alcaligenes 5	3.0	1.2	11.6	84.2
Alcaligenes 6	0.1	1.0	10.9	88.0
Pseudomonas 7	0.0	2.4	12.0	85.6

[a] Expressed as per cent of added DDM.

[b] Includes the amount of products not identified by GC-MS and also the amount of DDM not accounted for during the GC-analysis.

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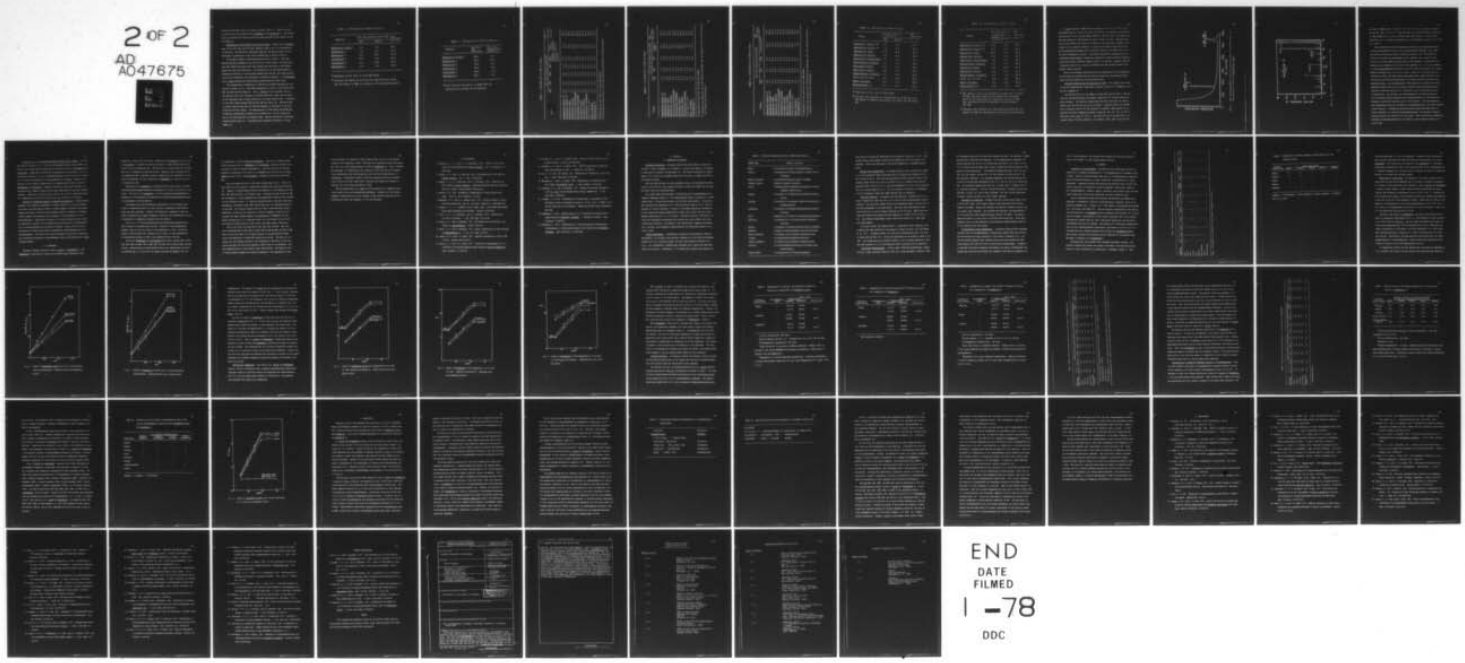

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identified from DBH with the six bacteria tested (Table 6). DPB was produced in particularly large amounts with Pseudomonas 4 and Alcaligenes 5. No products could be isolated from reaction solutions containing DBP as sole source of carbon (Table 7).

Cometabolism of DDT metabolites by various fungi. Except for M. alternans, none of the test fungi had ever been exposed to DDT or any of its metabolites or analogues. The extraction techniques used were the same as above, so the efficiency of extraction of the various metabolites is listed in Table 2.

The products formed in DDT metabolism are given in Table 8. DDD, DDE, DBH and DBP were produced by only four of the 12 fungi tested. No other products were identified except that some radioactivity was found in the aqueous phase left after the extractions. The radioactivity in the aqueous phase indicated the formation of water-soluble metabolites from DDT, but these products could not be detected by the techniques of extraction employed. P. brefeldianum and A. niger produced more water-soluble products than any other fungi.

The cometabolism of metabolites of DDT and the products detected are presented in Tables 9 to 12. Five fungi produced one or more of the products DDM, DBH, and DBP from DDA (Table 9). With A. conicus as test organism, 55.1% of the DDA was found to be converted to products which were either not extracted by the techniques used or bound strongly by the fungal myceliat mat. A majority of the fungi tested produced DBH and DBP from DDM (Table 10). DBP was formed in greater amounts than DBH, and PCPA was detected in treatments in solution containing the smut fungus. The identification of PCPA during the metabolism of DDM was accomplished by comparing its retention on a 3% OV-1 column with that of the TMS derivative of authentic PCPA. DBP was identified in solutions containing DBH (Table 11). No products were generated from DBP by 11 fungi (Table 12).

TABLE 6. Cometabolism of DBH by bacteria.

Organism	Per cent products detected[a]		DBH recovered, per cent
	DBP	Other[b]	
Pseudomonas putida 1	2.7	1.1	96.2
Pseudomonas 3	1.8	3.2	95.0
Pseudomonas 4	5.0	19.2	75.8
Alcaligenes 5	4.8	0.0	97.0
Alcaligenes 6	0.0	10.4	89.6
Pseudomonas 7	1.8	0.4	97.8

[a] Expressed as per cent of total DBH added.

[b] Includes the amount of products not identified by GC-MS and the amount of DBH not accounted for during GC-analysis.

TABLE 7. Cometabolism of DBP by bacteria.

Organism	Per cent DBP recovered	Per cent unidentified products [a]
Pseudomonas putida 1	96.1	3.9
Pseudomonas 3	89.2	10.8
Pseudomonas 4	95.4	4.6
Alcaligenes 5	90.8	9.2
Alcaligenes 6	98.2	1.8
Pseudomonas 7	100.2	0.0

[a] Also includes the amount of added DBP not accounted for during the GC-analysis.

TABLE 8. Cometabolism of DDT by fungi.

Fungus	Per cent products detected[a]						DDT recovered, per cent
	DBH	DBP	DDE	DDD	Water soluble unidentified products[b]	Other unidentified products	
Aspergillus flavus 141	0.6	0.0	0.6	1.5	0.0	0.7	96.6
Aspergillus flavus 147	0.0	0.0	0.0	1.8	0.0	0.0	99.5
Aspergillus niger	0.0	0.0	0.0	0.0	7.5	4.9	87.6
Aspergillus conicus	0.0	0.0	0.0	0.0	0.0	9.6	90.4
Penicillium brefeldianum	0.0	0.0	0.0	0.0	11.2	13.4	75.4
Pycnidiophora dispersa	0.0	0.0	0.0	0.0	3.8	4.2	92.0
Cercospora oryzae	0.0	0.0	0.0	0.0	0.0	1.2	98.8
Pyricularia oryzae	0.0	0.0	0.0	0.0	2.8	8.2	89.0
Thanatephorus cucumeris	0.0	3.0	3.8	0.1	2.3	6.4	84.4
False smut of rice	0.0	0.0	0.8	0.4	0.8	4.6	93.4
Mucor alternans	0.0	0.0	0.0	0.0	1.2	1.0	97.8
Schizophyllum sp.	0.0	0.0	0.0	0.0	0.0	9.0	91.0

[a] Expressed as per cent of total DDT added.

[b] Calculations were based on the radioactivity detected in aqueous phase.

TABLE 9. Cometabolism of DDA by fungi.

Fungus	Per cent products detected[a]				DDA recovered, per cent
	DDM	DBH	DBP	Unidentified[b]	
Aspergillus flavus 141	0.0	0.0	0.0	15.0	85.0
Aspergillus flavus 147	0.0	0.0	0.0	7.6	92.4
Aspergillus niger	0.6	0.0	3.8	14.6	81.0
Aspergillus conicus	8.3	0.0	0.0	55.1	36.6
Penicillium brefeldianum	2.6	0.0	0.0	6.4	91.0
Pycnidiophora dispersa	0.0	0.0	0.0	10.2	89.8
Cercospora oryzae	1.6	0.0	0.0	16.2	82.2
Pyricularia oryzae	0.0	0.0	0.0	19.2	80.8
Thanatephorus cucumeris	0.0	0.0	0.0	15.6	84.4
False smut of rice	0.0	0.0	0.0	5.6	94.4
Mucor alternans	0.0	0.0	0.0	0.4	99.6
Schizophyllum sp.	5.0	2.4	9.6	17.2	65.8

[a] Expressed as per cent of total DDA added.

[b] Includes the products not identified by GC-MS and the amount of DDA not accounted for during the GC-analysis.

TABLE 10. Cometabolism of DDM by fungi.

Fungus	Per cent products detected[a]				DDM recovered, per cent
	DBH	DBP	PCPA	Unidentified[b]	
Aspergillus flavus 141	0.0	0.0	0.0	11.4	88.6
Aspergillus flavus 147	0.1	1.4	0.0	9.9	88.6
Aspergillus niger	0.2	3.3	0.0	6.5	90.0
Aspergillus conicus	0.0	6.0	0.0	16.5	77.5
Penicillium brefeldianum	0.6	2.1	0.0	8.9	88.4
Pycnidiophora dispersa	0.0	7.8	0.0	15.4	76.8
Cercospora oryzae	0.0	3.2	0.0	9.0	87.8
Pyricularia oryzae	0.0	0.0	0.0	10.0	90.0
Thanatephorus cucumeris	0.0	0.0	0.0	8.6	91.4
False smut of rice	0.4	0.6	1.2	10.0	87.8
Mucor alternans	0.0	2.8	0.0	2.2	95.0
Schizophyllum sp.	2.0	7.6	0.0	3.6	86.8

[a] Expressed as per cent of total DDM added.

[b] Includes the products not identified by GC-MS and also the amount of DDM not accounted for during the GC-analysis.

TABLE 11. Cometabolism of DBH by fungi.

Fungus	Per cent products detected [a]		DBH recovered, per cent
	DBP	Other[b]	
Aspergillus flavus 141	0.0	0.0	101.6
Aspergillus flavus 147	1.8	6.5	91.7
Aspergillus niger	0.6	14.8	84.6
Aspergillus conicus	0.0	7.2	92.8
Penicillium brefeldianum	1.5	6.8	91.7
Pycnidiophora dispersa	0.0	1.4	98.6
Cercospora oryzae	0.0	3.4	96.6
Pyricularia oryzae	0.0	3.2	96.8
Thanatephorus cucumeris	2.4	3.8	93.8
False smut of rice	0.0	6.6	93.4
Mucor alternans	0.0	1.0	99.0
Schizophyllum sp.	4.0	4.2	91.8

[a] Expressed as per cent of DBH added.

[b] Includes the products not identified by GC-MS and also the amount of DBH not accounted for during the GC-analysis.

TABLE 12. Cometabolism of DBP by fungi.

Fungus	Per cent products detected		DBP recovered, per cent
	Product of R_t 171 s[a]	Other[b]	
Aspergillus flavus 141	0.0	1.6	98.4
Aspergillus flavus 147	0.0	4.8	95.2
Aspergillus niger	35.2	6.3	58.5
Aspergillus conicus	0.0	4.0	96.0
Penicillium brefeldianum	0.0	5.4	94.6
Pycnidiophora dispersa	0.0	5.2	94.8
Cercospora oryzae	0.0	10.8	89.2
Pyricularia oryzae	0.0	6.4	93.6
Thanatephorus cucumeris	0.0	0.0	101.4
False smut of rice	0.0	3.4	96.6
Mucor alternans	0.0	9.4	90.6
Schizophyllum sp.	0.0	5.6	94.4

[a] The amount of this new product was expressed in terms of DBP. The peak area of the product was measured from the GC trace and the equivalent amount of DBP was calculated from the standard curves prepared for DBP.

[b] Includes the products not identified by GC-MS and the amount of DBP not accounted for during the GC-analysis.

A. niger produced a compound with R_t value of 171 s on a 3% OV-1 column programmed from 110 to 180 C at a rate of 20 C/min. The amount of the product detected was 35.2% of the DBP added initially. The GC trace of the sample and the mass spectrum of the product are given in Figs. 9 and 10. The mass spectrum of the product was similar to an extent to the spectrum of authentic DPM, except for the unusually large $M^+ + 1$ peak and the peaks at m/e 139 and 141. p-Chlorophenylglyoxaldehyde with a molecular weight of 168 might give a similar mass fragmentation pattern. The product detected was suspected to be p-chlorophenylglyoxaldehyde, because the production of DPM by reductive dechlorination and further reduction seemed unlikely. In addition, authentic DPM had a retention time of 96 s, which is different from the retention time of the product formed from DBP.

None of the products detected during the cometabolism of DDT metabolites by the fungi was isolated from the controls containing uninoculated mineral salts solutions amended with the DDT metabolites.

Products formed during the degradation of PCPA. Two products were found in the gas chromatograms, these having retention times of 554 (compound A) and 653 sec (compound B).

The TMS derivatives of the sample in ether were analyzed with a Finnigan 3300 gas chromatograph-mass spectrometer supplied with a Systems Industries data processor. The operating temperatures and the column were the same as before, but the flow rate of N_2 was 18 ml/min. Combined GC-MS of the product with a retention time of 653 sec (product B) revealed a small parent ion at m/e 330 with major fragmentation peaks at m/e 198, 183, 163, 147, 93, and 73 and minor ones at m/e 133 and 117. The mass spectrum of the peak with a retention time of 554 sec (product A) also showed a small peak at m/e 330 and

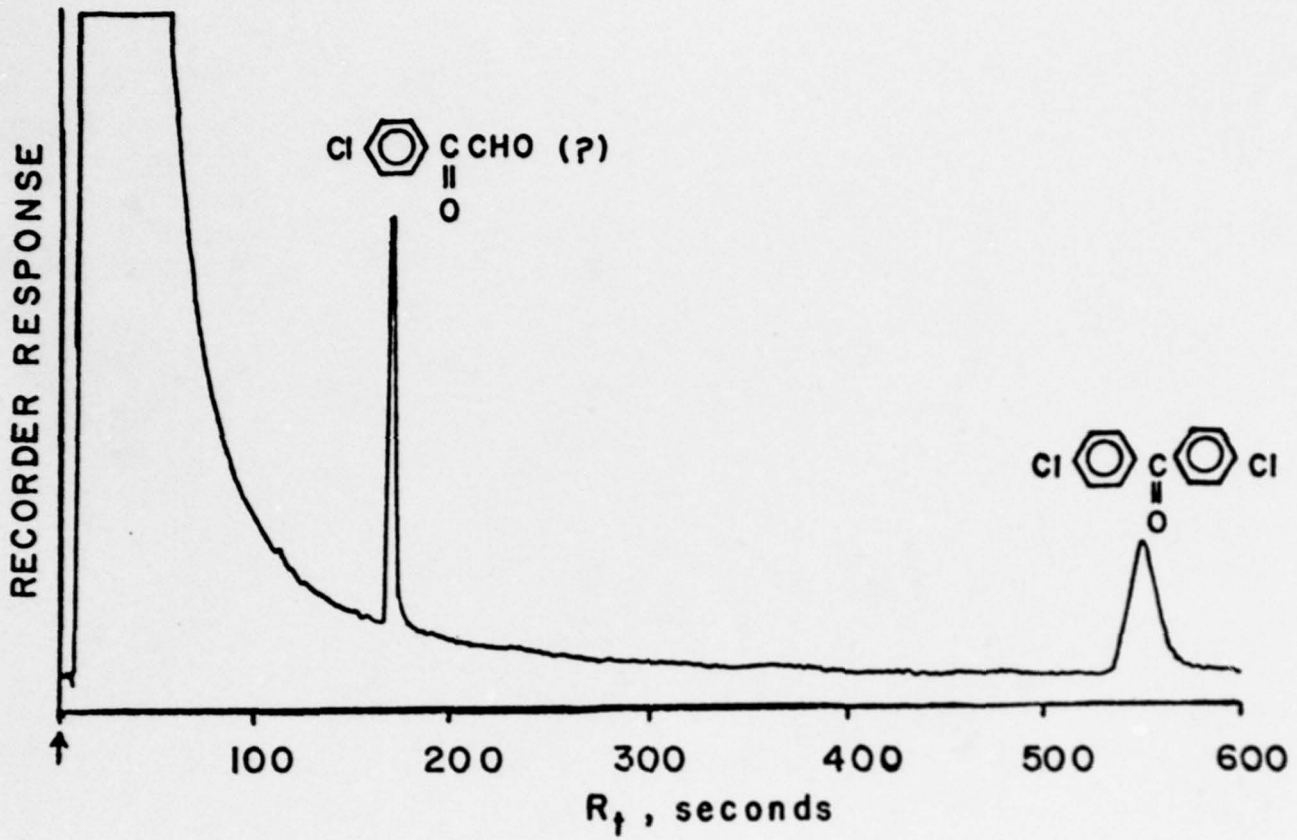

FIG. 9. Gas chromatographic trace showing the product formed during the cometabolism of DBP by Aspergillus niger.

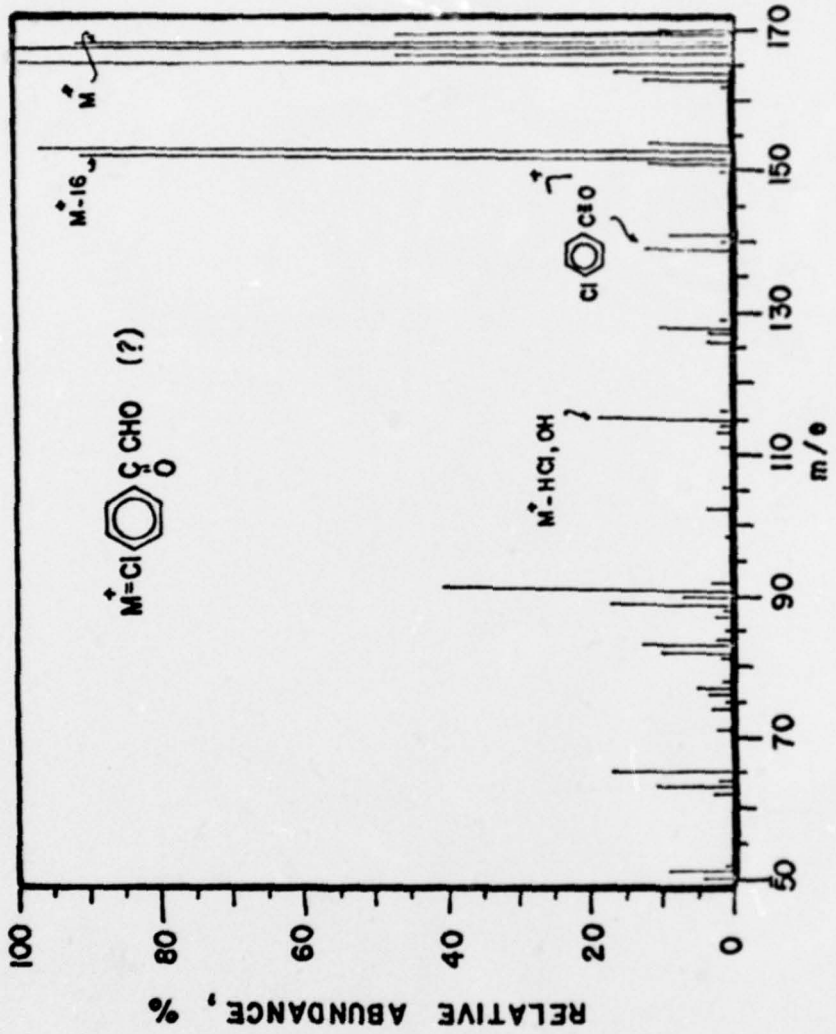

FIG. 10. Mass spectrum of a product formed from DBP.

had major fragment peaks at m/e 178, 163, 147, 94, and 73 plus minor fragments at m/e 287, 198, 133, and 117. Both molecules also contained chlorine inasmuch as they showed the 3:1 ratio of isotope peaks characteristic of chlorinated molecules; the P + 2 peaks were prominent for the fragments at m/e 198, 163, 147, 117, and 73.

These metabolites might be monohydroxy products of p-chlorophenylacetate (either p-chloromandelic acid or the ring-hydroxylated compound), the di-TMS derivatives of which would have a molecular weight of 330. The di-TMS derivative of authentic p-chloromandelic acid, however, had a retention time of 401 s and a mass spectrum unlike those of the unknowns. To determine the location of the hydroxyl on the ring of the unknowns, 0.5 to 1.0 g of authentic o- and m-hydroxyphenylacetic acids in 30 ml of 4 N HCl was treated with a small quantity of $KMnO_4$ and 1.0 to 2.0 g of $AlCl_3$. After 2 h, the mixture was extracted with ether, the ether extract was washed three times with water, and the resulting solution was concentrated in a flash evaporator and dried over anhydrous Na_2SO_4. Gas chromatography of TMS derivatives of these chlorinated compounds revealed that the sole product prepared from m-hydroxyphenylacetic acid had a retention time of 653 s, identical to that of the TMS derivative of metabolite B. By contrast, gas chromatography of the TMS derivative of the chlorinated products prepared from o-hydroxyphenylacetic acid showed the presence of peaks with retention times of 540 and 605 s. The mass spectra of the chlorinated derivative of authentic m-hydroxyphenylacetic acid and metabolite B were identical. Although the microbial product might have been either 4-chloro-2-hydroxy- or 4-chloro-3-hydroxyphenylacetate, the synthetic compound prepared could be the latter but not the former. Thus, the bacterium apparently oxidizes p-chlorophenylacetate by introducing a single hydroxyl group on C-3 of the ring.

Isolating 1,1,1',1'-tetra(p-chlorophenyl)dimethyl ether (DCBHE). Attempts were made to isolate 1,1,1',1'-tetra(p-chlorophenyl)dimethyl ether (DCBHE) suspected to be formed during the degradation of DDM by strains of Pseudomonas and Alcaligenes. DCBHE may be synthesized microbiologically from DDM, as its nonchlorinated analogue (1,1,1',1'-tetraphenyldimethyl ether) was earlier isolated by us during the degradation of diphenylmethane by P. putida. DCBHE was not detected in the products formed during the cometabolism of DDM by strains of Pseudomonas and Alcaligenes. DCBHE was chemically synthesized from DBH, and the detection of DCBHE in ether extracts was accomplished by gas-liquid chromatography. DCBHE has a retention time of 2050 s on a 3% OV-1 column maintained at a temperature of 250°C and a carrier gas (N_2) flow rate of 80 ml/min.

Isolation of organisms capable of metabolizing biphenyls. Polychlorinated biphenyls are frequently encountered in natural waters and, following biomagnification, have detrimental effects on higher members of natural food chains. Attempts were made to isolate microorganisms capable of growing on or metabolizing biphenyl and analogues such as biphenyl, 4-chlorobiphenyl, and 4,4'-dichlorobiphenyl. Many bacteria capable of utilizing biphenyl as sole source of carbon were isolated, but no organisms were obtained with 4-chlorobiphenyl or 4,4'-dichlorobiphenyl as sole source of carbon. Resting cells of one of the bacterial isolates were found to cometabolize 4-chlorobiphenyl and 4,4'-dichlorobiphenyl. The color of the reaction medium turned yellow, suggesting ring cleavage and formation of semialdehydes, which are characteristic of meta-cleavage enzymes.

C. DISCUSSION

Bacterial isolates obtained on DPM (P. putida 2, Enterobacter 2, and Pseudomonas 3) were able to oxidize only nonchlorinated analogues of DDT

metabolites, such as DPM, BH and BP. The bacteria (Pseudomonas strains 4 and 7, Alcaligenes 5) isolated on BH as sole source of carbon metabolized BH and BP but failed to metabolize DPM. Resting cells of various bacterial isolates were able to cometabolize DDM, DBH and DDA. However, these organisms failed to cometabolize DBP, a metabolic product formed during the degradation of DDT. Focht and Alexander (9) also reported that Pseudomonas sp. failed to grow on or cometabolize DBP.

Washed cells of Pseudomonas 3, obtained on DPM as sole source of carbon, oxidized p-hydroxydiphenylmethane and consumed 10.5 µmoles of 0_2/µmole of substrate. The ability of Pseudomonas 3 to oxidize p-hydroxydiphenylmethane in preference to DPM suggested the possibility of p-hydroxydiphenylmethane being an intermediate of DPM metabolism.

The present data show that Warburg respirometry is an excellent technique for screening bacteria for their ability to cometabolize DDT metabolites and their analogues. However, the method has a drawback in that one cannot distinguish the oxygen consumption resulting from cometabolism or from a stimulation of endogenous respiration by the chemical.

Feil et al. (7) detected hydroxylated and methoxylated metabolites of DDT in excreta of chickens fed with DDT. Formation of such hydroxylated products might be the first step to rapid degradation of DDT. During the present investigations with 6 bacteria and 12 fungi, no such hydroxylated or methoxylated products of DDT or its metabolites were detected.

Strains of Pseudomonas and Alcaligenes were found to convert DDT to DDD, DDE, DDM, DBH, and DBP; DDA to DDM, DBH, and DBP; DDM to DBH and DBP; and DBH to DBP. Identification of these products during the cometabolism of DDT and its metabolites is in line with the pathway proposed by Wedemeyer (18) for

the degradation of DDT by Klebsiella pneumoniae. DBP was not further metabolized by resting cells of Pseudomonas and Alcaligenes, thereby indicating the resistance of DBP to further biodegradation. The view that DBP is nonbiodegradable is supported by the lack of 0_2 consumption by resting cells of Pseudomonas strains 1 and 7 and Alcaligenes 5 incubated in solutions containing DBP.

Fungi can metabolize DDT (1) and other insecticides (14,15). Fungi are also known for their ability to hydroxylate randomly many pesticidal molecules (4). However, no hydroxylated products were detected in the metabolism of DDT, DDA, DDM, DBH and DBP by 12 different fungi. Conversion of DDT to DDE and DDD by four species of fungi suggested that the metabolism of DDT may have occurred via two different routes: (i) reductive dechlorination to form DDD, which was subsequently converted to DDA, DDM, DBH and DBP; and (ii) dehydrochlorination to form DDE. The reductive dechlorination of DDT to form DDD under aerobic conditions (created by the incubation of fungi on shakers) could not be explained, but these findings support the observations of Wedemeyer (18,19), Barker and Morrison (2,3), Hicks and Corner (11), and others (6,12,13) that the reductive dechlorination of DDT occurs also under aerobic conditions.

Four of the fungi converted DDT to DDD, DDE, DBH, and DBP. The fungi also transformed DDA to DDM, DBH, and DBP; DDM to DBH and DBP; and DBH to DBP. This is the first report of the identification of various products formed during the degradation of known DDT metabolites by fungi. The data also indicate that the enzymes involved in the conversion of DDT occur in the organisms even when they were never exposed to DDT or any of its analogues. DDM was cometabolized by a smut fungus to DBH, DBP and PCPA. The identification of these products suggest two different pathways for the degradation of DDM

by this fungus: (i) oxidation of DDM to DBH and DBP; and (ii) ring cleavage leading to the formation of PCPA. PCPA was first reported by Focht and Alexander (8,9) as a ring cleavage product of DDM by Pseudomonas sp. Later, Pfaender and Alexander (16) demonstrated the formation of PCPA when DDT was incubated first anaerobically and then aerobically with cell-free preparations of a pseudomonad. Considerable amounts of DBP had accumulated during the metabolism of DDM by the fungi tested here, indicating the absence of enzymes responsible for the further degradation of DBP.

DBP was metabolized by A. niger with the production of a compound tentatively identified as p-chlorophenylglyoxaldehyde. Formation of this product suggests a mechanism for the ring cleavage of DBP different from the one hypothesized by Focht and Alexander (9) for DDT analogues.

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V. Section 5

A. MATERIALS AND METHODS

Enrichment procedures. Seventeen insecticides were added as either sole sources of carbon, phosphorus or nitrogen in a liquid salts medium. The medium is described by Alexander and Lustigman (2). The inoculum consisted of approximately 5% of either soil randomly mixed from five sites or sewage collected at the primary effluent.

When the pesticide was the sole phosphorus source, the glassware was washed with a 20% (vol/vol) HNO_3 cleaning solution, rinsed five times with tap water and seven times with deionized water.

The insecticides used represent a broad spectrum of organophosphorus and carbamate compounds (Table 1). The compounds were added to solutions to 50 ppm insecticide-P, 150 ppm insecticide-N, and 1000 ppm insecticide-C. The enrichment cultures were incubated in 16 X 100 mm screw-cap tubes at 29 C. Upon appearance of visible turbidity, a loopful of enrichment medium was transferred to the liquid salts medium amended with 2% Noble agar (Difco) and the corresponding insecticide as C, N, or P source. Following isolation, individual colonies were repeatedly reintroduced into similar but sterile media until pure cultures were obtained. The bacteria were identified by comparing morphological, cultural, and biochemical characteristics with described bacteria (8,11, 42,54, 55,56).

Growth measurements. Turbidimetric readings were performed by removing periodically 3 ml aliquots from cultures incubated in 125 ml baffled Erlenmeyer flasks at 29 C on a Gyrotory shaker (150 rpm) (New Brunswick Scientific Co., N.J.). All turbidimetric readings were performed with a Bausch and Lomb Spectronic 20 at 420 nm. Alternately, 5 ml aliquots were removed from growing cul-

TABLE 1. Pesticidal compounds and their common abbreviations.

Common name	Chemical structure
Aspon	0,0,0,0-Tetra-n-propyl dithiopyrophosphate
Azodrin	0,0-Dimethyl-O-(2-methylcarbamoyl-1-methyl-vinyl)-phosphate
Baygon (Propoxur)	2-(1-Methylethoxy)phenol-methyl-carbamate
Carbaryl (Sevin)	N-Methyl-1-naphthyl carbamate
Carzol	(3-Dimethylamino-(methylene-iminophenyl))-N-methyl carbamate hydrochloride
Dasanit	0,0-Diethyl O-[4-(methylsulfinyl)phenyl]phosphorothioate
Diazinon	0,0-Diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate
Dimethoate	0,0-Dimethyl-S-(N-methylcarbamoylmethyl)phosphorodithioate
Dylox	Dimethyl(2,2,2-Trichloro-1-hydroxyethyl)phosphonate
Malathion	O,O-Dimethyl-S-(1,2-dicarbothoxyethyl)phosphorodithioate
Mesurol	3,5-Dimethyl-4-(methylthiolphenol-methyl-carbamate
Methoxyl	S-Methyl-N-((methylcarbamoyl)oxy)thioacetimidate
Methyl parathion	O,O-Dimethyl-O-p-nitrophenol phosphorothioate
Orthene	O,S-Dimethylacetylphosphoramidothioate
(Ethyl) Parathion	O,O-Diethyl-O-p-nitrophenyl phosphorothioate
Trithion	S-((-p-Chlorophenylthio)methyl)O,O-diethyl phosphorodithioate
Vapcna (DDVP)	2,2-Dichlorovinyl-O,O-Dimethylphosphate

tures and cell protein was determined by the method of Lowry et al. (39). Crystalline bovine serum aubumin (Nutritional Biochemical Corp.) was used as the standard. Protein was monitored at 660 nm and compared to a standard protein curve.

Resting cells preparations. To prepare resting cells, cultures were grown in 1-liter Erlenmeyer flasks containing 500 ml of medium and incubated at 29 C and 150 rpm for 48 h. The cells were harvested by centrifugation (Sorvall centrifuge, model RC2-B) for 15 min at 10,000 X g at 4 C, washed thrice with Na-barbitol buffer (0.04 M, pH 7.2), and then resuspended in 25 ml of the same buffer to an optical density of 1.5. To 5 ml aliquots of resting cell suspension in 25 ml Erlenmeyer flasks were added 300-450 µg/ml of substrate.

Cell-free extracts. To prepare cell-free extracts, cultures from 1.5 liters of medium were grown for 48 h at 29 C and 150 rpm and harvested by centrifugation. The cells were washed thrice with Na-barbitol buffer (0.04 M, pH 7.2) and then resuspended in the same buffer. The cells were sonicated with four 1-min bursts at 6 C with a Branson Sonicator set at 40 watts. Whole cells and large cell debris were removed by centrifugation at 12,000 X g for 15 min at 4 C. The resulting supernatant fluids, which were stored at 0 C, were used for enzyme assays.

To purify further the enzyme-containing supernatant fluid, $(NH_4)_2SO_4$ fractionation was used according to the methods of Carman and Leyin (10) and Dawson et al. (15). Protamine sulfate (2%, Nutritional Biochemicals Corp.) was used to remove RNA and DNA (10). Protein was determined by the method of Lowry et al. (39). The enzyme assay was started by adding 1.0 ml of enzyme preparation (300-400 µg/ml protein) to 5 ml of Na-barbitol buffer containing 500 µM substrate).

Gas-liquid chromatography. A Perkin-Elmer 3920B gas-chromatograph, equipped with a flame ionization detector (FID) and a flame photometric detector (FPD)

with phosphorus and sulfur filters and recorder was used. Two different columns and operating conditions were employed. For organophosphorus compounds, the packing material was 3% OV-17 on 100/120 mesh Gas Chrom Q in a 1.83 m X 2 mm (i.d.) Teflon-lined stainless steel column (columns and packing materials from Applied Science Laboratory). The operating temperatures were: 140, 200, and 230 C for the column; 190 and 245 C for the injector; and 250 and 275 C for the interface (detector). For carbamate compounds, the packing material was 5% SE-30 on 80/100 mesh Chromosorb W (HP) in a 1.83 m X 2 mm (i.d.) glass column. The operating temperatures were 160 C, column; 240 C, injector; and 275 C, interface (detector). For FID, the gas flows were: 30 ml/min, carrier gas (helium); 50 psig, air; and 21 psig, hydrogen. For FPD, the gas flows were: 30 ml/min, helium; 60 psig, air; and 29 psig, hydrogen.

Extraction of substrate. Aliquots from the cultures were placed in 160 X 100 mm test tubes, and an equal volume of pesticide grade ethyl acetone (Fisher) was added. Tubes were vortexed, the contents allowed to settle, and the organic layer decanted. The procedure was repeated, and approximately 2 g of an analytical grade anhydrous Na_2SO_4 (Fisher) was added. The organic phase was stored in 2 dram vials with Teflon-lined screw-caps. Based upon a standard curve for each substrate, two extractions removed greater than 95% of the substrate from the aliquot.

Derivatization using diazomethane. To determine water-soluble breakdown products from the organophosphates, aliquots were removed from the cultures and derivatized with CH_2N_2 according to the method of Daughton et al. (13). The derivatized products were extracted twice with ethyl acetate and anhydrous Na_2SO_4 over glass wool prior to gas-liquid chromatography. Standards of dimethyl and diethyl phosphate and dimethyl and diethyl thiophosphate compounds were similarly derivatized (All standards from American Cyanamide Co.).

Prior to derivatization, the aliquots were treated with 10% cold trichloroacetic acid (Fisher) to stop further enzyme activity.

B. RESULTS

Isolation of microorganisms. Enrichment-culture techniques involved exposing soil or sewage microorganisms to 17 organophosphorus or carbamate insecticides. Bacteria isolated because they grew on 10 organophosphorus compounds were purified and studied as to their versatility in metabolizing 12 organophosphates. Organisms isolated from diazinon and malathion were the most versatile, being able to metabolize 10 and 9 compounds, respectively (Table 2). The organophosphates---azodrin, dasanit, malathion, and orthene---were the most widely used while trithion and vapona were the least frequently used.

The two organisms isolated from diazinon and malathion enrichments were subjected to biochemical, cultural, and morphological diagnostic tests as described by Skerman (54). Stanier et al. (56), Manual of Microbiological Methods (55), and Colwell and Wiebe (11). The two isolates corresponded to the description and definition of Pseudomonas given by Doudoroff and Palleroni (8) in being strictly aerobic, gram-negative, motile (polar flagellum) rods, that produced catalase, were oxidase positive, had a respiratory metabolism, and had growth requirements that were not complex. The presence of a fluorescent pigmentation, lack of poly-β-hydroxybutyrate accumulation, and growth at 4 but not at 41 C indicated that the organism isolated from diazinon was Pseudomonas putida. The organism isolated from malathion was not identified to the species level and hence is referred to as Pseudomonas 7.

Organisms were also selected from carbamate-enrichment cultures. The bacteria isolated from baygon and carbaryl enrichments were purified and studied as to their versatility in metabolizing 5 carbamates (Table 3). Both

TABLE 2. Metabolism of various organophosphorus insecticides by soil and sewage isolates.

Organism isolated from	Insecticide tested											
	Aspon	Azodrin	Dasanit	Diazinon	Dimethoate	Dylox	Malathion	Methyl Parathion	Orthene	(Ethyl) Parathion	Trithion	Vapona
Aspon	++	++	+	++	++	-	++	-	++	+	-	-
Azodrin	+	++	++	+	+	-	+	+	++	-	-	-
Dasanit	-	++	++	-	+	+	+	+	++	-	-	+
Diazinon	+	++	++	++	+	++	++	++	++	+	-	-
Dylox	-	++	++	+	+	++	++	-	+	-	-	-
Malathion	+	++	++	-	+	-	++	++	++	+	-	+
Methyl Parathion	-	+	+	+	-	-	+	+	-	+	-	-
Orthene	+	++	++	+	-	-	+	-	++	-	-	-
(Ethyl) Parathion	-	-	++	+	+	+	++	-	++	+	-	-

Symbols: - No turbidity; + light turbidity; ++ heavy turbidity. Incubated for 72 h at 29 C.

TABLE 3. Metabolism of various carbamate insecticides by soil and sewage isolates.

Organism isolated from	Insecticide tested				
	Baygon	Carbaryl	Carzol	Mesurol	Methomyl
Baygon	++	++	+	+	-
Carbaryl	++	++	+	+	-
Carzol	-	-	+	-	-
Mesurol	+	-	-	+	-
Methomyl	-	+	-	-	+

Symbols: - No turbidity; + light turbidity; ++ heavy turbidity. Incubated for 72 h at 29 C.

isolates metabolized 4 of the five compounds. Diagnostic tests performed on these isolates indicated that they were identical and belonged to the genus Pseudomonas. The isolate will be referred to as Pseudomonas 44. Enrichment experiments indicated that none of the isolates could use the organophosphorus or carbamate compounds as: the sole source of carbons; as carbon and phosphorus sources; or as carbon and nitrogen sources.

Metabolism of insecticides. To determine if the three isolates were metabolizing the insecticide compounds and not inorganic contaminant phosphorus or nitrogen, growth experiments were conducted. Both P. putida and Pseudomonas 7 (data not shown) showed a linear relation between maximum optical density (420 nm) and P-substrate concentration up to 0.15 mM P (Fig. 1). Maximum cell yield occurred with KH_2PO_4, while diazinon and malathion produced approximately 65 and 70% of the cell yield compared to KH_2PO_4. Based upon the carbon in the medium, a P-concentration of 0.15 mM gave approximately a 15:1 (C:P) ratio. A 15:1 ratio of C:P approximates the cellular C:P ratio of bacteria. Thus, the P in the medium was limiting up to 0.15 mM.

Similarly, the growth of Pseudomonas 44 was found to be linear with respect to N-substrate concentration (Fig. 2). The maximum cell yield occurred at 0.75 mM N and with $(NH_4)_2SO_4$. The cell yield with baygon and carbaryl as N source was approximinately 75% of the maximum cell yield. Based upon the carbon concentration in the medium, 0.75 mM N represented a 4:1 (C:N) ratio, which approximates that of cellular C:N. Thus, N concentration of 0.75 mM was limiting. Additionally, the data show that the bacteria were not oligophosphorophiles or oligocarbophiles. Similar results were obtained at different pH values (6.5-8.5) and temperatures (20-45 C).

To demonstrate further that the bacteria were utilizing the compounds as P or N sources, cell growth (as cell protein) was correlated with substrate

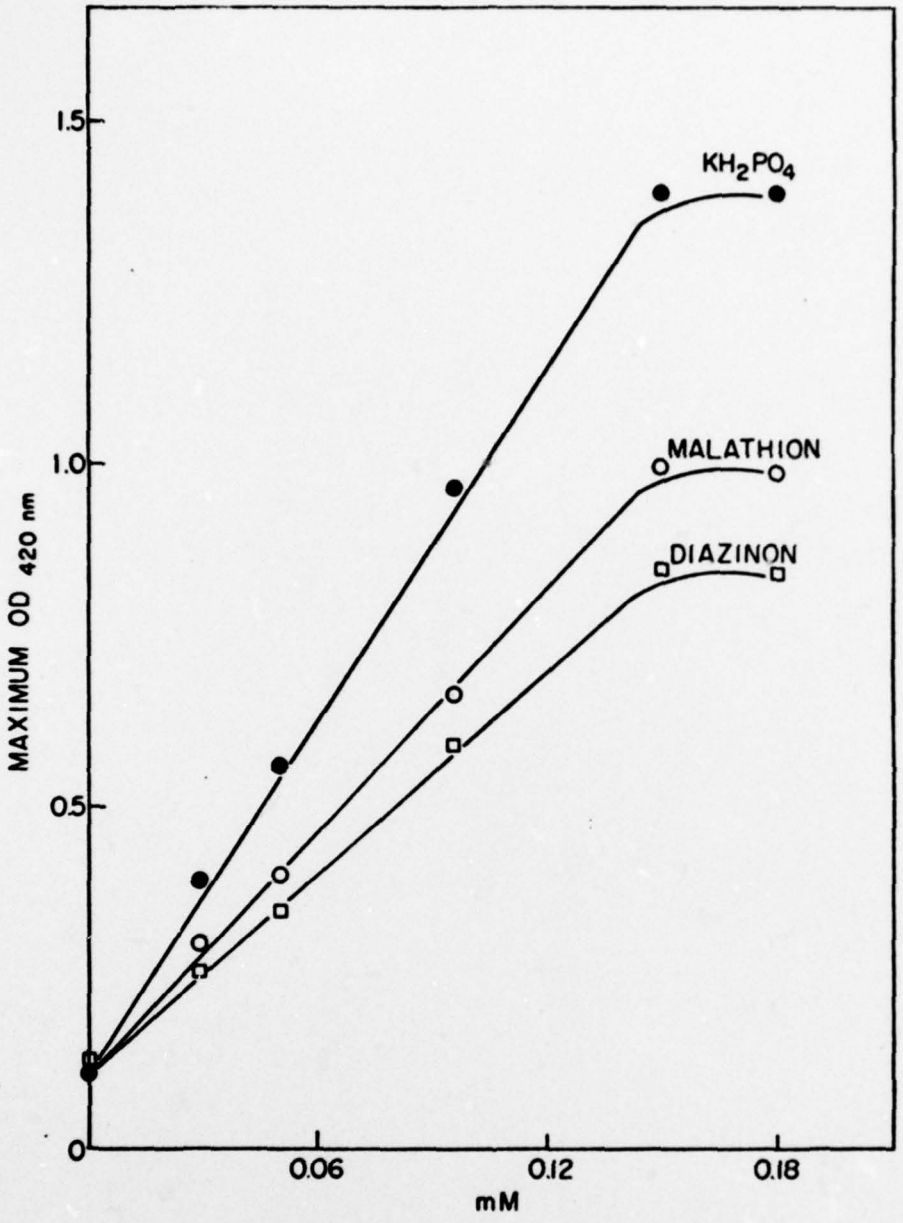

Fig. 1. Growth of Pseudomonas putida after 48 h at various substrate concentrations. Substrates were sole phosphorus source.

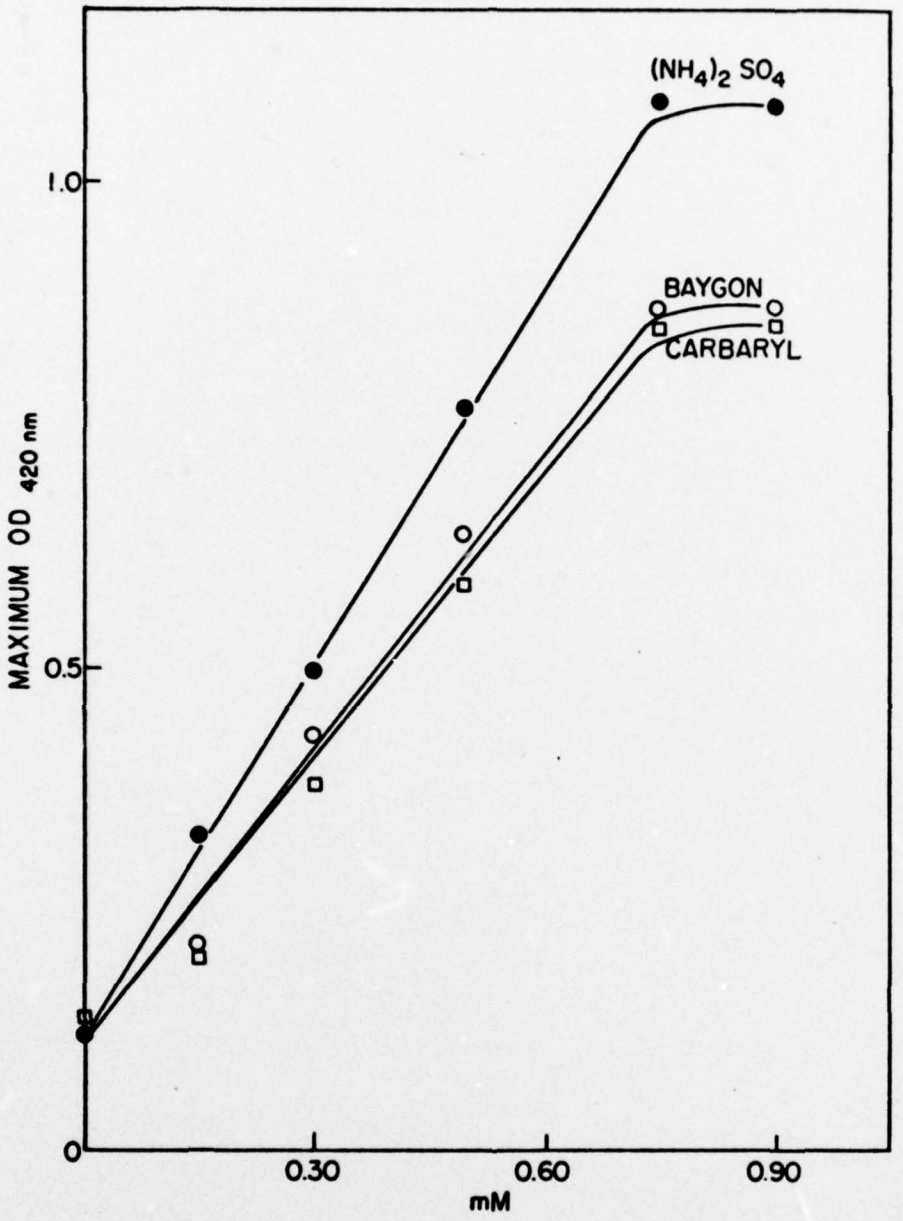

Fig. 2. Growth of Pseudomonas 44 after 48 h at various substrate concentrations. Substrates were sole nitrogen source.

disappearance. The growth of P. putida and the disappearance of diazinon and malathion were linear with respect to time (Fig. 3). This linearity indicates that at any time during the growth cycle, the rate and amount of P utilized (as determined by % P in the substrate (10%) as well as substrate disappeared) agreed closely with the theoritical rate and amount of P needed by the cells for growth as determined by cell protein and the relationship of P to cell protein (2% of cell protein is P)(1). Similar results were obtained with Pseudomonas 7 (Fig. 4).

The rate of growth of Pseudomonas 44 correlated also with the rate of substrate disappearance (Fig. 5). Growth (cell protein) and substrate disappearance were linear with respect to time throughout the growth cycle. The amount of N utilized (as determined by % N in baygon and carbaryl (7%)) approached the theoretical amount of N needed by the cells for growth as determined by cell protein and the relationship of N to cell protein (16% of cell protein is N)(1). Thus, P. putida and Pseudomonas 7 metabolized diazinon and malathion as sole P source and Pseudomonas 44 metabolized baygon and carbaryl as sole N source. The organisms were not utilizing contaminant inorganic PO_4 or NH_4 as P or N sources in place of the insecticidal compounds. Further support for this conclusion was obtained when colorimetric analyses of the organophosphorus and carbamate compounds indicated the absence of detectable inorganic PO_4 and NH_4.

Resting-cell suspensions. The ability of P. putida and Pseudomonas strains 7 and 44 to metabolize their respective abovementioned insecticidal compounds raised the questions whether the metabolism was enzyme mediated, and if so, was the enzyme system induced or constitutive. The questions were answered with resting-cell suspensions.

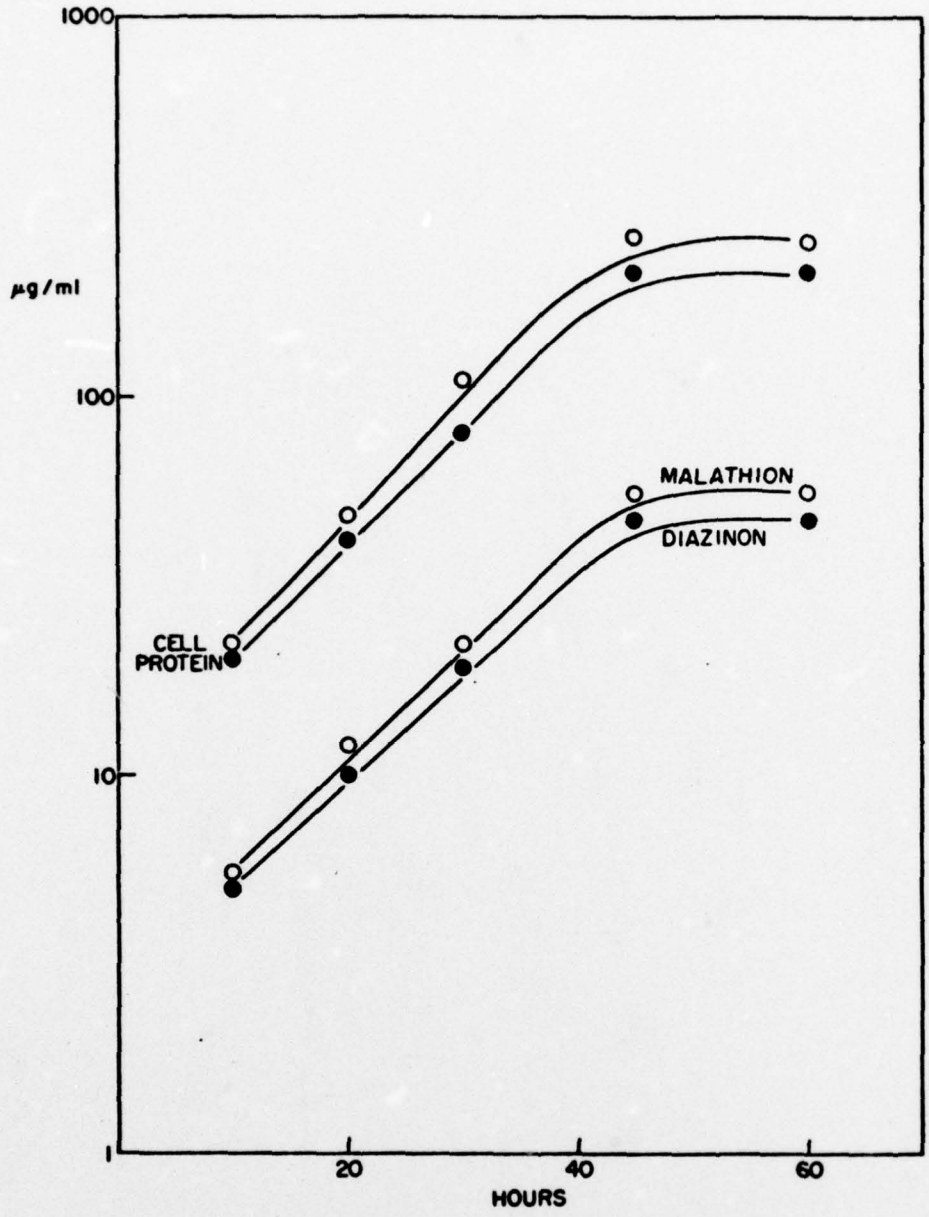

Fig. 3. Growth of Pseudomonas putida and disappearance of 65 µg/ml (0.2 mM) diazinon and malathion. Compounds were sole phosphorus source.

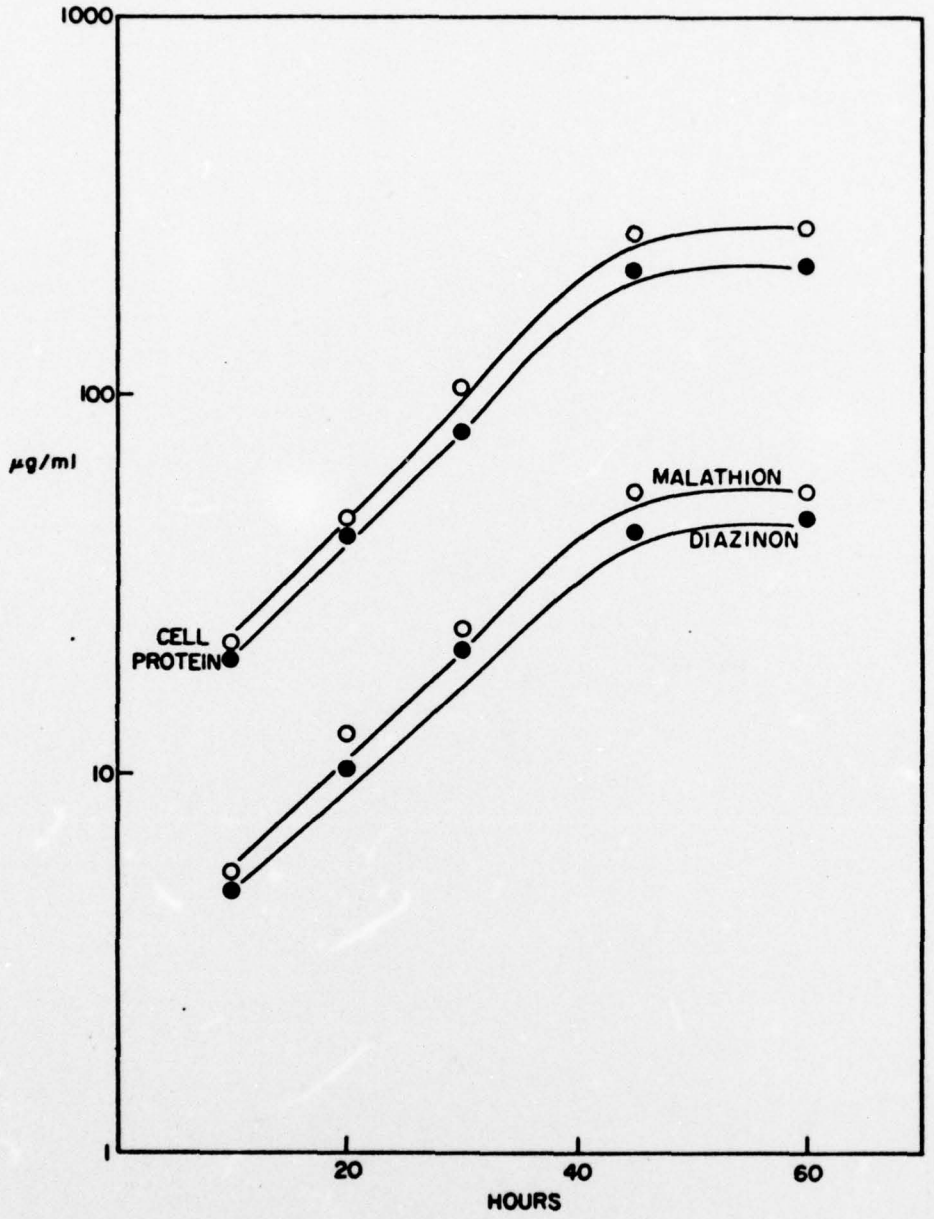

Fig. 4. Growth of Pseudomonas 7 and disappearance of 65 µg/ml (0.2 mM) diazinon and malathion. Compounds were sole phosphorus source.

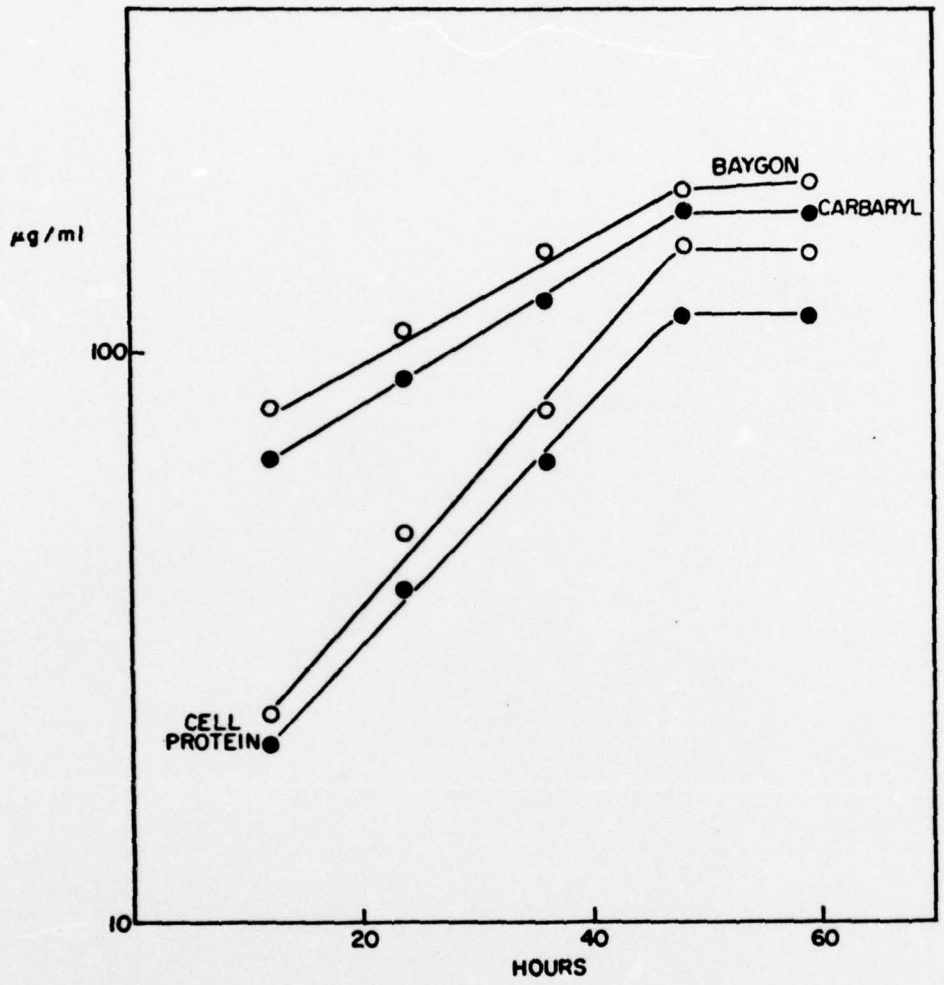

Fig. 5. Growth of Pseudomonas 44 and disappearance of 210 µg/ml (1.0 mM) baygon and carbaryl. Compounds were sole nitrogen source.

When P putida was grown in inorganic PO_4, diazinon and malathion, only diazinon-grown resting-cell suspensions metabolized diazinon (Table 4). In contrast, malathion was metabolized by all resting-cell suspensions regardless of the P source in the growth medium. Chloramphenicol added to the resting cells prior to the substrate did not alter the results. No significant degradation of diazinon and malathion by boiled cells or in sterile buffer occurred. The data suggest an enzymatic basis for substrate disappearance. Further, the metabolism of diazinon appears to be mediated by an induced enzyme system, while malathion metabolism involves a constitutive enzyme system. Similar results were obtained using resting cell suspension of Pseudomonas 7 (Table 5).

When Pseudomonas 44 was grown in inorganic NH_4, baygon, and carbaryl and resting cell suspensions prepared, only cells grown in baygon and carbaryl metabolized these two carbamates (Table 6). Chloramphenicol did not alter the results. The lack of significant degradation of baygon and carbaryl by NH_4-grown cells, boiled cells, and in sterile buffer suggests an induced enzyme system is responsible for metabolism of the two carbamates. This system is present in the cells prior to the addition of the substrates. The similarity in the metabolism of baygon and carbaryl by baygon- and carbaryl-grown cells suggests a similar induced enzyme system for both compounds.

Cell-free extracts. To determine further the enzymatic activity as well as the substrate specificity of the induced and constitutive enzyme systems, cell-free extracts from the three bacteria were prepared.

The specific activity and substrate specificity for P. putida extracts partially purified by $(NH_4)_2SO_4$ fractionation are shown in Table 7. The constitutive enzyme system from cell-free extracts of PO_4- and malathion-grown cells showed activity on 9 of 12 organophosphorus compounds. The specific activities ranged from 0.23 to 1.80 μM substrate disappeared/min/mg protein.

TABLE 4. Disappearance of diazinon[1] and malathion[1] effected by resting-cell suspensions[2] of Pseudomonas putida.

P source in growth medium	Chloramphenicol added[3]	Concn, µg/ml		
		Viable cells		
		Diazinon	Malathion	Boiled cells[4]
KH_2PO_4	+	13(4.3)[5]	277(92)	
	-	11(3.7)	256(85)	8(2.7)
Diazinon	+	273(91)	236(79)	
	-	293(98)	259(86)	9(3.0)
Malathion	+	13(4.3)	271(90)	
	-	17(5.7)	282(94)	9(3.0)

[1]Initial concentration: 300 µg/ml.

[2]Optical density (420 nm): 1.5. Incubated for 36 h at 29 C and 150 rpm.

[3]Chloramphenicol concentration: 100 µg/ml.

[4]Cells boiled for 15 min prior to substrate addition. Numbers refer to the mean of the values obtained with diazinon and malathion, treated and untreated, with chloramphenicol.

[5]Represents % of initial substrate concentration. Diazinon and malathion in sterile Na-barbitol buffer (pH 7.2) had a mean disappearance of 9 µg/ml (3.0%) in 36 h.

TABLE 5. Disappearance of diazinon and malathion* effected by resting-cell suspensions of Pseudomonas 7.

P source in growth medium	Chloramphenicol added	Concn, µg/ml		
		Viable cells		
		Diazinon	Malathion	Boiled cells
KH_2PO_4	+	11(3.7)	244(81)	
	-	15(5.0)	243(81)	9(3.0)
Diazinon	+	259(86)	257(86)	
	-	278(92)	268(89)	11(3.7)
Malathion	+	14(4.7)	246(82)	
	-	11(3.7)	270(90)	10(3.3)

*See footnotes to Table 4.

TABLE 6. Disappearance of baygon[1] and carbaryl[1] effected by resting-cell suspensions[2] of Pseudomonas 44.

N source in growth medium	Chloramphenicol added[3]	Concn, µg/ml		
		Viable cells		
		Baygon	Carbaryl	Boiled cells[4]
$(NH_4)_2SO_4$	+	31(6.9)[5]	28(6.2)	
	-	35(7.7)	30(6.7)	28(6.2)
Baygon	+	379(84)	353(78)	
	-	410(91)	396(88)	31(6.9)
Carbaryl	+	382(85)	346(77)	
	-	393(87)	383(85)	32(7.1)

[1]Initial concentration: 450 µg/ml.

[2]Optical density: 1.5. Incubated for 36 h at 29 C at 150 rpm.

[3]Chloramphenicol concentration: 100 µg/ml.

[4]Cells boiled prior to substrate addition. Numbers refer to the mean of the values obtained with baygon and carbaryl, treated and untreated with chloramphenicol.

[5]Represents % of initial substrate concentration. Baygon and carbaryl in sterile Na-barbitol buffer (pH 7.2) had a mean disappearance of 28 µg/ml (6.2%) in 36 h.

TABLE 7. Specific activity and substrate specificity of cell-free extracts[1] of Pseudomonas putida.

P source in growth medium	Specific activity on various substrates[2] (μM substrate disappeared/min/mg protein)											
	Diazinon	Malathion	Azodrin	Dasanit	Dylox	Orthene	Aspon	Dimethoate	Methyl Parathion	(Ethyl) Parathion	Trithion	Vapona
KH_2PO_4	0.05	1.13	1.38	1.22	0.05	1.41	0.53	0.39	0.31	0.23	0.05	0.44
Diazinon	1.82	1.21	1.73	1.84	1.53	1.97	0.62	0.51	0.48	0.39	0.03	0.04
Malathion	0.08	1.80	1.20	1.56	0.06	1.79	0.79	0.44	0.63	0.28	0.07	0.35
Boiled extract[3]	0.10	0.03	0.11	0.03	0.13	0.07	0.03	0.06	0.07	0.04	0.10	0.08
Pronase-treated extract[4]	0.05	0.09	0.07	0.08	0.12	0.10	0.11	0.05	0.07	0.06	0.09	0.08

[1]Partially purified by $(NH_4)_2SO_4$ fractionation; protein concentration: 400 μg/ml. Incubated for 45 h at 29 C.

[2]Initial concentration: 150 μg/ml.

[3]Boiled for 5 min.

[4]Pronase concentration: 15 μg/ml. Numbers from boiled and pronase treatments represent mean of values obtained with extracts of KH_2PO_4-, diazinon-, and malathion-grown cells. Substrates in sterile buffer had a mean disappearance of 7 μg (4.7% of initial conc.) in 45 h.

The induced enzyme system (diazinon-grown cells) demonstrated activity on 10 of 12 compounds with specific activities ranging from 0.39 to 1.97 μM substrate disappeared/min/mg protein. The specific activities represent a 4-5 fold increase over that in the crude cell-free extract. Pronase-treated and boiled cell-free extracts had no activity, and the substrate in sterile buffer was stable. Thus, both constitutive and induced enzyme systems demonstrated a broad substrate specificity for organophosphorus compounds. The data also reaffirm that diazinon is metabolized by an induced enzyme system, while malathion is metabolized by a constitutive enzyme system. With the exception of dylox, which appeared to be metabolized by a constitutive enzyme system, the specific activities and substrate specificity for cell-free extracts of Pseudomonas 7 were very similar to those for P. putida (Table 8).

The specific activity and substrate specificity for Pseudomonas 44 are shown in Table 9. No activity as compared to the controls occurred when the substrates were tested with a cell-free extract from NH_4-grown cells. However, specific activities for 4 carbamates varied from 0.29 to 1.98 μM substrate disappeared/min/mg protein with cell-free extracts from baygon- and carbaryl-grown cells. Thus, with Pseudomonas 44 only a broad substrate-specific induced enzyme system was capable of metabolizing the carbamates studied. The broad substrate specificity of the cell-free extract is compatible with the versatility demonstrated by whole cells on various insecticidal compounds.

Determination of metabolic breakdown products of organophosphates. Chemical and enzymatic hydrolysis of organophosphorus compounds generally results in the formation of an ionic dialkyl (thio)phosphorus ester (5,37,41,47). To determine if such ionic dialkyl esters were formed by P. putida and Pseudomonas 7, the cell-free extracts were analyzed. After derivatizing a sample with CH_2N_2 and extracting with ethyl acetate, aliquots of the sample were analyzed in the

TABLE 8. Specific activity and substrate specificity of cell-free extracts of Pseudomonas 7.

P source in growth medium	Specific activity on various substrates (μM substrate disappeared/min/mg protein)											
	Diazinon	Malathion	Azodrin	Dasanit	Dylox	Orthene	Aspon	Dimethoate	Methyl Parathion	(Ethyl) Parathion	Trithion	Vapona
KH_2PO_4	0.07	1.27	1.83	1.11	1.35	1.44	1.87	0.22	0.38	0.67	0.03	0.07
Diazinon	1.73	1.49	1.60	1.22	1.30	2.01	0.31	1.41	0.41	1.01	0.11	0.13
Malathion	0.11	1.36	1.65	1.08	1.41	1.66	1.71	0.28	0.32	0.50	0.08	0.09
Boiled extract	0.08	0.13	0.11	0.06	0.05	0.04	0.06	0.05	0.04	0.07	0.11	0.12
Pronase-treated extract	0.07	0.07	0.12	0.03	0.11	0.11	0.07	0.13	0.07	0.03	0.09	0.08

See footnotes to Table 7.

TABLE 9. Specific activity and substrate specificity of cell-free extracts[1] of Pseudomonas 44.

N source in growth medium	Specific activity on various substrates[2] (μm substrate disappeared/min/mg protein)				
	Baygon	Carbaryl	Carzol	Mesurol	Methomyl
$(NH_4)_2SO_4$	0.11	0.08	0.13	0.05	0.09
Baygon	1.98	1.77	0.36	0.39	0.11
Carbaryl	1.71	1.80	0.45	0.29	0.06
Boiled extract[3]	0.05	0.10	0.09	0.08	0.04
Pronase-treated extract[4]	0.06	0.12	0.13	0.07	0.05

[1]Partially purified by $(NH_4)_2SO_4$; protein concentration: 300 μg/ml. Incubated for 45 h at 29 C.

[2]Initial concentration: 100 μg/ml.

[3]Boiled for 5 min.

[4]Pronase concentration: 15 μg/ml. Numbers from boiled and pronase treatments represent mean of values obtained with extracts of $(NH_4)_2SO_4$-, baygon-, and carbaryl-grown cells. Substrates in sterile buffer had a mean disappearance of 6 μg (6% of initial conc.) in 45 h.

GLC using FPD. The retention times of metabolites were compared to retention times of dimethyl phosphate, dimethyly thiophosphate, diethyl phosphate, and diethyl thiophosphate.

Of the 8 organophosphorus insecticides studied, 7 were converted to anionic dialkyl (Table 10). Dimethyl phosphate was liberated from azodrin and dylox; dimethyl thiophosphate from malathion and orthene; diethyl phosphate from diazinon; and diethyl thiophosphate from dasanit, diazinon, and (ethyl) parathion. Vapona was not converted to ionic esters. The liberation of ionic dialkyl (thio)phosphates indicates that, at least in P. putida and Pseudomonas 7, the metabolic pathway of organophosphorus metabolism is similar. The data suggest also that the cell-free extracts contain a phosphatase (phosphotriesterase) responsible for the cleavage of the ionic dialkyl phosphate ester.

Since P. putida and Pseudomonas 7 liberated ionic diakyl phosphate and thiophosphate compounds, an experiment was conducted to determine if these two organisms could metabolize representative dialkyl phosphate esters. The basal salts medium was amended with 0.2 mM phosphorus as trimethyl phosphate (TMP), dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), trimethyl thiophosphate (TMTP), triethyl phosphate (TED), diethyl phosphate (DEP), diethyl thiophosphate (DETP), triethyl thiophosphate (TETP), or tri-n-butyl phosphate (TBP). No growth occurred with DMP, DEP, DMTP, DETP, TMTP, or TETP (Fig. 6) (Pseudomonas 7 data not shown). However, with TMP, TEP and TBP, both organisms grew and reached optical densities of approximately 1.3, 1.2, and 1.0, respectively. The inability of P. putida and Pseudomonas 7 to metabolize DMP, DEP, DMTP, DETP, TMTP, or TETP suggests: (a) that ionic phosphorus esters cannot be used as P source, and (b) that compounds with sulfur are toxic to the organisms.

TABLE 10. Production of ionic dialkyl (thio)phosphorus esters from various organophosphorus insecticides by Pseudomonas putida and Pseudomonas 7.

Insecticide	Ionic dialkyl phosphorus ester			
	Dimethyl phosphate	Dimethyl thiophosphate	Diethyl phosphate	Diethyl thiophosphate
Azodrin	+	-	-	-
Dasanit	-	-	-	+
Diazinon	-	-	+	+
Dylox	+	-	-	-
Malathion	-	+	-	-
Orthene	-	+	-	-
(Ethyl)parathion	-	-	-	+
Vapona	-	-	-	-

Symbols: +: present; -: not detected

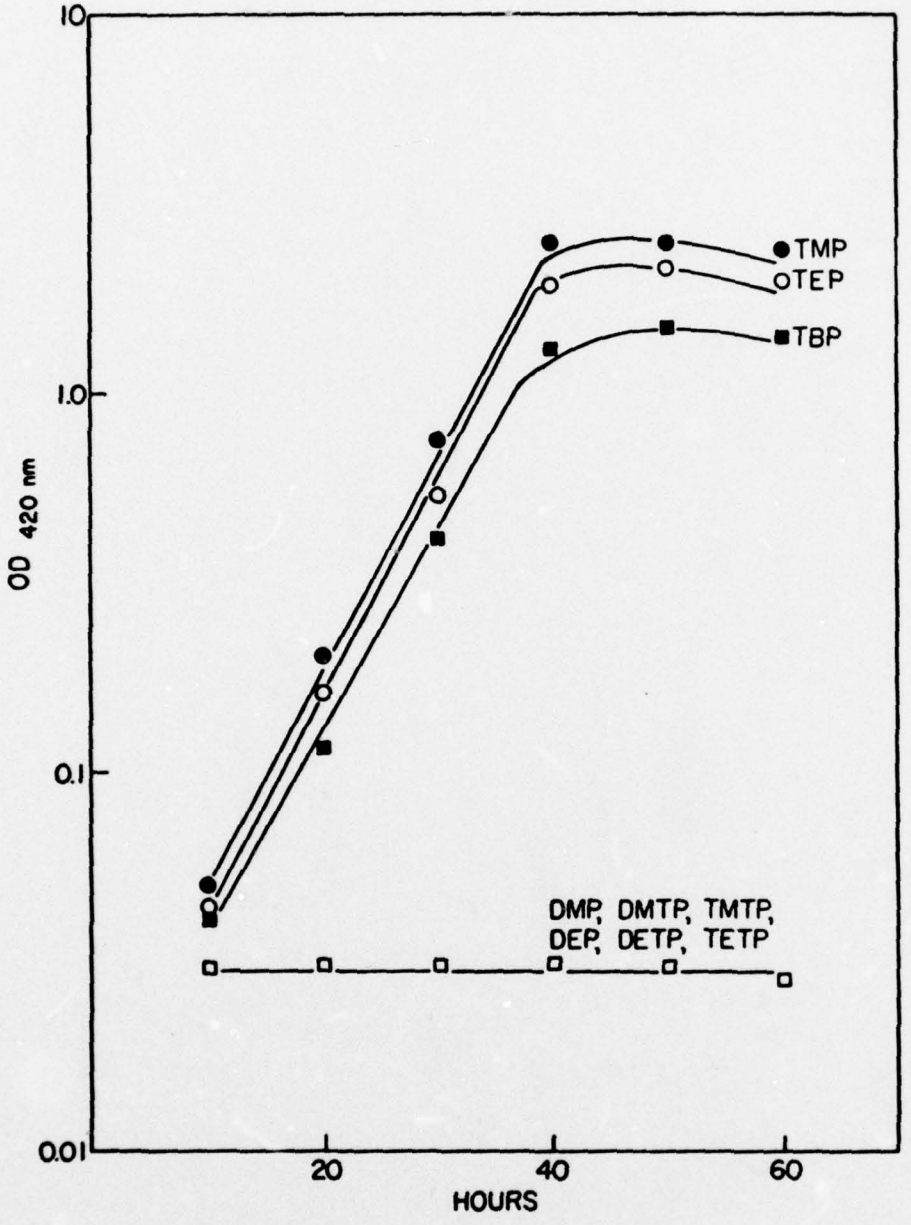

Fig. 6. Growth of Pseudomonas putida using various substrates at 0.2 mM as sole phosphorus source.

C. DISCUSSION

Bacterial isolates were obtained that could use a variety of organophosphorus and carbamate compounds as their sole sources of P and N, respectively. The P substrate-metabolizing organisms were identified as Pseudomonas putida and Pseudomonas 7, while the N substrate-metabolizing organism was identified as Pseudomonas 44.

P. putida and Pseudomonas strains 7 and 44 utilized the insecticidal compounds as sole sources of P or N and were not utilizing either inorganic PO_4 or NH_4 as P or N sources. Studies of resting cell suspensions of these organisms indicated that the breakdown of diazinon, malathion, baygon, and carbaryl was enzymatic (rather than chemical) and resulted from both induced and constitutive enzymes. Adaptative and constitutive enzyme systems have been proposed as mechanisms for the development of effective pesticide-degrading soil populations (32). Numerous articles in the literature report soil microorganisms active in degrading organophosphate and carbamate (5,6,9,14,25,28,32,34, 37,40,41,47).

The partially purified cell-free preparation from P. putida and Pseudomonas 7 growing on KH_2PO_4, diazinon, and malathion as sole P source had a broad substrate specificity and metabolized other organophosphorus insecticides. This broad substrate specificity is in line with the versatility of the organisms to metabolize various organophosphates. The specific activities varied from 0.23 to 2.01 μM substrate disappearance/min/mg protein. In general, the activity for both organophosphates and carbamates correlated with the extent and rate of growth of whole cells using the corresponding substrate as a P or N source. When metabolic studies were conducted with the organophosphorus compounds, various ionic dialkyl (thio)phosphate esters were found, indicating

enzymatic hydrolysis at the aryl P-O bond. This view is supported by various reports in the literature (6,14,20,21). The enzyme specificity for other insecticides was less influenced by alkyl substitutions on the phosphorus atom than by aromatic ring substitution or changes.

The ability of crude cell-free extracts (derived from a bacterium using one organophosphate) to act on a number of substrates has been demonstrated by Munnecke (44,45). He noted that a crude enzyme preparation from a mixed culture growing on parathion had a broad substrate specificity for other organophosphates. Although he did not conduct studies to identify the metabolic products, he believed that enzymatic hydrolysis occurred at the aryl P-O bond. Thus, the literature concerning organophosphate metabolite production by cell-free extracts is scare.

The broad substrate specificity of cell-free enzymes was evident with carbamate compounds also. Induced enzymes from baygon- and carbaryl-grown cells demonstrated specific activities ranging from 0.29 to 1.98 μM substrate disppeared/min/mg protein for baygon, carbaryl, carzol, and mesurol. Cell-free enzyme extracts from a number of adapted microorganisms have been shown to degrade various other pesticides of the same class. Thus, the crude extract of Arthrobacter sp. grown on 2,4-dichlorophenoxyacetic acid hydrolyzed a wide range of related chlorinated phenols (38). Kearney (33) purified an enzyme (from Pseudomonas sp. grown on isopropyl-N-(3-chlorophenyl)carbamate) which hydrolyzed related phenylcarbamates and several acylanilide herbicides. Engelhardt et al. (18,19) using Bacillus sphaericus grown on the phenylurea herbicide, linuron, found that induced cells produced an acylamidase capable of hydrolyzing several other phenylureas and acylanilides. Thus, many soil microorganisms demonstrate a versatility in metabolized various classes of pesticidal compounds.

One of the principle chemical reactions mediated by soil microorganisms for the breakdown of organophosphates and carbamates is hydrolysis (5,9,37, 41,47,52). Pesticides which are attacked initially by a hydrolytic mechanism are relatively short-lived in soil (5,9,47, 52). Generally, three enzyme classes are involved in this hydrolysis of amide and ester linkages: carboxy-esterase and phosphatase for organophosphates (Table 11), and carboxy-esterase and amidase for carbamates (Table 12).

Although carboxyesterases are the principle enzymes involved with many organophosphates (6,12,20,24,25,28,40,47) results of the present study suggest that the cell-free extracts from P. putida and Pseudomonas 7 contain specific phosphatases, or more correctly phospholipases or phosphotriesterases, since phosphatases are not active against phosphorus triesters and prefer phosphates rather than phosphorothionates as substrate (43). However, Heuer et al. (30) showed disappearance of guthion, parathion, pyrimiphosmethyl catalyzed by acid phosphatases.

The phospholipases may be intimately involved in the initial attack on organophosphates and thiophosphates by microorganisms (14). These enzymes could have appreciable significance in the hydrolysis of organophosphates in natural environments, especially in soil, since it has recently been shown that phospholipase production can be a major characteristic of soil actinomycetes (36). The premise that phospholipases are involved in the initial hydrolytic attack on organophosphorus insecticides is further supported by the fact that phospholipases can act at the supersubstrate interface of non-water-soluble substances; in fact, hydrolysis rates for water-soluble substances are often much slower (7). Phospholipases may have further involvement in organophosphate hydrolysis reactions, since the activities of some carboxyesterases and phosphotriesterases greatly resemble the activities of certain phospholipases (14,49).

TABLE 11. Generalized structure and degradation of organophosphorus insecticides.

$(RO)_2P(S)Z$	$(RO)_2P(O)Z$
Phosphorothioate	Phosphate
R: CH_3 or C_2H_5; Z: leaving group	
$(RO)_2P(S)OX \rightarrow (RO)_2P(O)OX$	Activation
$(RO)_2P(O)SX \rightarrow (RO)_2P(O)OH + HSX$	Phosphatase
$(RO)_2P(S)OX \rightarrow (RO)(OH)P(S)OX$	Dealkylation
$RCOOR' \rightarrow RCOOH + R'OH$	Carboxyesterase

TABLE 12. Generalized structure and degradation of carbamate insecticides

$CH_3(R)NCOOX$			
R: H or CH_3; X = substituted phenol or a heterocyclic or aromatic enol			
$CH_3(R)NCOO(X)$	→	$CH_3(R)NCOOH$ + XOH	Carboxyesterase
$CH_3(R)NCOOX$	→	HOCOOX + $CH_3(R)NH$	Amidase

Since it is generally accepted that phosphorylated compounds do not pass freely through cell membranes, Rockman and Heppel (51) concluded that the hydrolysis of non-penetrating nucleotides was by enzymes located external to the cytoplasmic membrane. The cell wall would be a logical locale for phospholipases, especially in gram-negative bacteria, since the wall contains lipopolysaccharide and phospholipids in which covalent bonding (i.e., triesters) may be predominant (16).

The criterion for substrate specificity by the crude enzyme extracts in this report was the disappearance of the substrate. Coincident with this disappearance was the appearance of the ionic dialkyl (thio)phosphate particular to the organophosphate studied. The presence of these ionic dialkyl phosphates suggests a similar pathway of degradation in P. putida and Pseudomonas 7 via hydrolytic attack on the parent compound. Since pesticides that undergo initial hydrolytic attack are relatively short-lived in soil (5,9,37,47,52) one can say that organophosphates (and carbamates) have a low persistence in the environment. Although this observation is generally correct, one cannot be lulled into a sense of complacency, for, with the hydrolysis of organophosphates, comes the accumulation of the respective ionic dialkyl(thio)phosphate.

Although DMP, DEP, DMTP, and DETP were found as metabolites of the various organophosphates studied, neither P. putida nor Pseudomonad sp. 7 could utilize DMP, DEP, DMTP, DETP, TMTP, or TETP as sole phosphorus source. In contrast, Wolfenden and Spence (59) reported the isolation of an Enterobacter aerogenes that could utilize DMP, but only as a sole phosphorus source. This is the only report of utilization of an ionic dialkyl phosphate by a defined microbial culture. Although the purity of the culture was suspect or mixed, others have reported instances of dialkyl phosphate utilization, but only as a sole phosphorus source (3,9,27,28,59, Tiedje, J. M. 1966. M. S. Thesis, Cornell University). However, similar to the present study, none of these

investigators could demonstrate the utilization of an ionic or nonionic alkyl thiophosphate as sole phosphorus source. Many thiophosphate compounds are known inhibitors of phosphatases (14,57).

The results indicate that ionic and nonionic alkyl thiophosphates (and in some instances ionic dialkyl phosphates) are exceedingly stable, not only to chemical hydrolysis, but also to attack by highly acclimated organophosphate-utilizing bacteria. The inability of P. putida and Pseudomonas sp. 7 to metabolize ionic dialkyl phosphates and ionic and nonionic alkyl thiophosphates was probably due to the lack, inaccessibility, or inhibition of a suitable phosphodiesterase or phosphotriesterase and the toxicity of the substrate. Thus, the breakdown and disappearance of the organophosphate parent compound could generate enormous amounts of potentially highly recalcitrant ionic dialkyl phosphates and thiophosphates in the environment. In addition, these compounds are used in very large quantities in several industries (14).

Although phosphodiesters occur naturally (e.g., nucleotide macromolecules and phospholipids (47) and are assimilated by microorganisms, little less is known about the man-made alkyl phosphates which are potential (and real) products of their parent organophosphorus insecticides. Ionic dialkyl phosphates and especially thiophosphates are exceedingly unreactive and highly unusual molecules to living organisms (17,23,35,46). They are not without toxicity to eucaryotes. Some ionic dialkyl phosphates are quite toxic to fish, either singly or synergistically with the parent compound (4) and to rats as an anticholinesterase agent (53). Plants have been shown to assimilate and convert ionic dialkyl phosphates to cholinesterase inhibitors (22,48). The photolysis of certain organophosphates and ionic dialkyl phosphates can yield trialkyl phosphates that have been shown to be potent potentiators of the toxicity toward warm-blooded animals by organophosphates which contain carboxylic ester groups (26,29,50,57).

In view of these results and the fact that many organophosphates undergo hydrolysis to yield the phosphodiesters, concern about the environmental fates of these ionic alkyl phosphates and thiophosphates seems justified. However, surprisingly little is known about their potential fate in nature. In addition, these results further support the idea that pesticide "nonpersistence" is too often equated simply with "disappearance" of the parent compound (31).

The pesticides examined were chosen because of their diverse dialkyl phosphate moiety and because many of them (both organophates and carbamates) are among the most commonly used insecticides in the United States (58). Thus, the cell-free extracts used in this study can hydrolyze an important portion of the marketed insecticidal compounds. Too little work, however, has been done concerning substrate specificity of induced or constitutive enzymes toward different chemical classes of, as well as structurally different, pesticides. The choice of a pesticide that serves as inducer is important since the pesticide will strongly determine the specific activity as well as the synthesis of the enzyme(s) for various other pesticides. For practical and environmental reasons, it would be advantageous to know if one pesticide will be metabolized by enzyme(s) induced by the presence of a different pesticide.

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Microbial degradation of various organochlorine, organophosphorus and carbamate pesticides by aquatic and soil microorganisms was studied. Bacteria, fungi, and an alga (Cylindrospermum sp.) metabolized DDT to water-soluble products. DDT, DDA, and 2-chlorosuccinic acid were not detected; however, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene, DDD, DDE, DDM, DBH, DBP and PCPA were detected. The degradation of DDT metabolites yielded: DDM, DBH and DBP from DDA; DBH and DBP from DDM; and DBH

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from DBP. Microorganisms were found capable of converting diphenylmethane, and analog of DDT, to 1,1,1',1'-tetraphenyldimethyl ether. Pseudomonas putida converted bis(p-chlorophenyl)acetic acid to bis(p-chlorophenyl)methane, 4,4'-dichlorobenzhydrol and 4,4'-dichlorobenzophenone by cometabolism. The organism also dehalogenated 4,4'-dichlorobenzhydrol and 4,4'-dichlorobenzophenone and converted diphenylmethane to benzhydrol and benzophenone as well as cleaving diphenylmethane and benzhydrol to phenylacetic and phenylglycolic acids, respectively. Arthrobacter sp. metabolized PCPA to 4-chloro-3-hydroxyphenylacetic acid. DDT and its breakdown products had no significant effect on respiration of microbial communities or algal productivity. The biodegradability of DDT analogs was related to their chemical structure. Studies of resting-cell suspensions and cell-free enzyme extracts of two organophosphate- and one carbamate-utilizing bacteria indicated that both constitutive and induced broad, substrate-specific enzymes were responsible for organophosphate and carbamate metabolism. The metabolites of various organophosphates were: dimethyl and diethyl phosphate and thiophosphate, indicating hydrolytic attack on the parent compound by phosphatases.

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